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In addition to separate chapters on each class of chlorinated aromatic compounds, reviews of microbial physiology, genetics, and methods of biodegradation assessment are included. One chapter reviews biodegradation of these compounds in scaled-up processes. Included also are potential biodegradation pathways for all classes of chloroaromatic compounds, an illustrated list of compounds, a glossary, a reference list, an additional bibliography, citation index, and a subject index.

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FOREWORD

Today's rapidly developing and changing technologies and industrial products and practices frequently carry with them the increased generation of solid and hazardous wastes. These materials, if improperly dealt with, can threaten both public health and the environment. Abandoned waste sites and accidental releases of toxic and hazardous substances to the environment also have important environmental and public health implications. The Hazardous Waste Engineering Research Laboratory assists in providing an authoritative and defensible engineering basis for assessing and solving these problems. Its products support the policies, programs and regulations of the Environmental Protection Agency, the permitting and other responsibilities of State and local governments and the needs of both large and small business in handling their wastes responsibly and economically.

This report is a compendium describing the current level of understanding of chlorinated aromatic compound decomposition by microbiological pathways. The halogenated aromatic compounds are one of the most persistent collections of chemicals contaminating the environment. The persistent nature of these chemicals is attributable to the inability of the environment to cleanse itself of these contaminants. Since microbiological communities are fundamental participants in the detoxification chain, the environment generally does not have microorganisms capable of degrading the halogenated aromatic compounds. This report specifically identifies microorganisms capable of degrading many of the halogenated organic species. In many cases, the substrate is tracked through a decomposition pathway to end product. Many factors contribute to the biodecomposition of a given chemical; among the most important are: the chemical nature of the substrate molecule and substituents, substrate concentration, environmental parameters, nutrient and growth factor availability, and the presence of organisms capable of degrading the substrate. However, insufficient information is currently available to assess the potential biodegradability of a substrate based on information generated under different environmental conditions.

David G. Stephan
Director
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ABSTRACT

This report was initiated because of a need to bring together a review of the literature pertaining to microbial metabolism of chlorinated aromatic compounds. The information gathered here is extensive although not exhaustive. Most attention has been given to reports of bacterial, fungal, and cyanobacterial pathways of substrate degradation where metabolites or end products have been identified. Studies which report data on metabolites arising from incubation of the substrate with mixed cultures or environmental samples and studies which show disappearance of the compound have also been evaluated and included.

In addition to separate chapters on each class of chlorinated aromatic compounds, reviews of microbial physiology, genetics, and methods of biodegradation assessment are included. One chapter reviews biodegradation of these compounds in scaled-up processes.

The potential biodegradation pathways for all classes of chloroaromatic compounds have been brought together into an overview diagram.

The review indicates that many factors are involved in assessing the biodegradability of a compound including the nature of the molecule, substrate concentration, environmental parameters, availability of nutrients and growth factors, and presence of degradative microorganisms. Not enough information is presently available to permit extrapolation from one environment to another or to utilize data on a similar compound to assess the biodegradability potential of a given substrate.

This report includes an illustrated list of compounds, a glossary, a reference list, an additional bibliography, citation index, and a subject index.

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ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

BOD —biochemical oxygen demand
COD —chemical oxygen demand
H_c —Henry's law constant
HRT —hydraulic residence time
MCRT —mean cell residence time
MLSS —mixed liquor suspended solids
MLVSS —mixed liquor volatile suspended solids
ppb —parts per billion (μg/l)
ppm —parts per million (μg/ml or mg/l)

Abbreviations of herbicides are noted at first mention and in index.

SYMBOLS

a —empirical constant in Langmuir isotherm
b —empirical constant in Langmuir isotherm
C_a —concentration of substrate in the mixed liquor
C_l —concentration of substrate in the liquid phase
C_s —concentration of substrate in the solid phase
f_L —weight fraction of lipid-like compounds in the biomass
K^b —a biological disappearance rate constant, first order in substrate concentration
K_f —empirical constant for the Freundlich isotherm equation
K_{ow} —octanol-water partition coefficient
K_s —Monod half-saturation constant
K^{st} —first order stripping rate constant
n —empirical constant in Freundlich isotherm equation
Q_{air}/V —ratio of the air flow rate to the hydraulic reactor volume
REM_b —percent removal of the substrate from the system by the biotransformation fate mechanism
REM_e —percent removal of the substrate from the system in the effluent
REM_s —percent removal of the substrate from the system by sorption on biomass
REM_{st} —percent removal of the substrate from the system by the stripping fate mechanism
X —concentration of biomass in the mixed liquor
Y —yield coefficient
μ —specific growth rate
μ_m —maximum specific growth rate

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SECTION 1
INTRODUCTION

The first synthesized organochloride compound, ethyl chloride, was prepared in about 1440, but large scale synthesis of industrially important chlorinated organics, including chlorinated aromatic compounds, occurred during only the past few decades (214a). In general, the chlorinated aromatics of industrial synthesis or byproducts thereof represent one class of xenobiotic recalcitrant compounds. These compounds have few or no naturally occurring structural analogs and are persistent or resistant to both biological and abiotic degradation. Many of the chlorinated aromatics share similar physical chemical properties of low water solubility and high K_{ow} (octanol-water partition coefficients) which suggest lipophilicity or bioaccumulation potential.

Properties such as persistence, bioaccumulation, and demonstrable chronic and acute toxicity to human and nonhuman animal populations cause immediate concern related to their environmental health effects and their potential for ecosystem perturbations on long- and short-term exposure. These concerns cause the frequent appearance of these chemicals on EPA priority pollutant lists and have led to extensive research on their fate in the environment and their potential for microbiological transformation to less hazardous molecules.

The term biodegradation has had many different meanings. The Biodegradation Task Force, Safety of Chemicals Committee, Brussels (299) has defined biodegradation as the molecular degradation of an organic substance resulting from the complex action of living organisms. A substance is said to be biodegraded to an environmentally acceptable extent when environmentally undesirable properties are lost. Loss of some characteristic function or property of the substance by biodegradation may be referred to as biological transformation. In this text, we have attempted to restrict use of the term biodegradation in favor of more specific terminology. In this respect, biotransformation refers to any alteration of an organic molecule by organisms, and mineralization means the transformation of an organic molecule to its inorganic component parts with release of halide, CO_2, and/or methane.

The potential for microbial transformation of chlorinated aromatic compounds is related to the two fundamental roles of heterotrophic microorganisms in the global ecosystem. Both roles relate to the central concept of microbial decomposition of organic matter to release stored energy in the organic molecules (whether natural or anthropogenic in origin) and to return essential nutrients, such as CO_2, to biogeospheric nutrient pools. The first has thermodynamic implications while the second relates to elemental and nutrient cycling. While these simplified generalizations apply to most naturally produced organic matter, certain aromatic polymers such as lignin are persistent in the environment. Factors contributing to persistence of organic compounds have been discussed previously (92a) and can be summarized as insolubility, large molecular size or polymeric nature, toxicity, and anthropogenic origin. Although most or all of these factors are important for various chlorinated aromatics, the anthropogenic origin of most chlorinated aromatics is critical for the prediction of the rate of elimination of the compound from the environment and its eventual fate.

In general, microbial decomposition or biodegradative capabilities have coevolved with the synthesis of organic matter by plants and animals over the millenia. However, in the case of many chlorinated organics of industrial synthesis, 40 to 50 years is an unlikely time frame to expect evolution of enzyme systems capable of decomposition of such compounds. Yet evidence has accumulated that some of these compounds can be biologically transformed and extensively biodegraded by a diversity of heterotrophic microorganisms.

Such evidence has arisen primarily from studies using pure and mixed microbial cultures and from lesser-controlled environmental fate experiments. This evidence has promoted much additional research on the molecular mechanism of biodegradation, which in turn has permitted studies leading to increased knowledge of more detailed aspects of biodegradation itself. Currently, major questions exist as to the rate at which biodegradation occurs in various environments and the potential for kinetic prediction of pollutant fate based on laboratory and environmental observations. Research needs directed at this major question have caused a renewed focus on individual microbial populations that may be specifically responsible for biodegradation of a narrow spectrum of chlorinated aromatic substrates, and on physical/chemical environmental parameters that may modulate both the population and their catabolic activity. Developments in these areas have led to the relatively recent detection of bacterial strains harboring extrachromosomal DNA (plasmids) that genetically encodes enzymes which mediate the biodegradation of specific groups of aromatic and chlorinated aromatic compounds. Coupled with the availability of new molecular genetic techniques, such as DNA probe technology, it has become feasible to plan research to examine catabolic gene maintenance and transfer in natural populations and to detect specific biodegradative microorganisms in the environment. Such development will lead to greater predictive capabilities on the long-term persistence of selected chlorinated aromatic pollutants, and will provide for insight in the evolution and reassortment of genes responsible for biodegradation. With these genetic techniques there is a potential therefore to enhance biodegradative capacity among natural populations.

This report begins with three sections that provide an overview of microbial physiology, genetic information transfer and processing, technologies for gene manipulation, and methods of biodegradation assessment. These principles are applicable to microorganisms in general and are specifically relevant to metabolism of chlorinated aromatic compounds. Readers wishing review of these basic microbiological concepts should read these chapters before turning to subsequent sections.

The next section includes a review of pathways of metabolism of non-chlorinated aromatic compounds from which the chlorinated pollutants are derived. The microbial metabolism of most of these compounds has been studied extensively and these data form the basis for an understanding of the biotransformation pathways of the more complex chlorinated aromatic molecules.

The following nine sections discuss the microbial metabolism of the chlorobenzoic acids, chlorobenzenes, chlorophenols, pentachlorophenol, chlorophenoxy and chlorophenyl herbicides, chlorinated biphenyls, DDT and related compounds, and chlorinated dioxins and dibenzofurans. These compound classes represent all of the major classes of chlorinated aromatic molecules with environmental pollution potential.

Each chapter details the information available on metabolism of these compounds by pure cultures or consortia of bacteria, cyanobacteria, and fungi. Emphasis has been placed on studies in which metabolites have been identified and, where possible, complete or partial pathways have been reported. Studies reporting evolution of CO_2 and chloride release have been reviewed and are discussed as well. The disappearance of these pollutants in soils, water, sewage, and other environments has also been noted, although the body of literature relating to herbicide disappearance is so extensive that it has only been summarized and has not been reviewed as completely as the other topics. The fate of these compounds in scaled-up biological treatment processes is the subject of the next section.

The final chapter summarizes the information reviewed here and draws together the pathways of metabolism of chlorinated aromatic molecules into an overview diagram indicating the potential for biodegradation of a compound under optimum conditions. This is an idealized representation, as few environmental situations would comprise all the factors necessary for complete biotransformation and/or mineralization of these recalcitrant compounds.

This report includes a list of citations and a bibliography of additional references, including several excellent reviews on the biodegradation of various classes of organic compounds. All of the compounds noted in this report, including metabolites, are registered in an illustrated alphabetical list in the appendix. A citation index and a subject index are also included. Finally, a glossary of many of the scientific terms that appear in the text is appended in order to aid the reader.

This document is intended to be a general reference for environmental decision-makers who are interested in the fate of chlorinated aromatic compounds with respect to microbial activity. It is also meant to provide a resource for scientists and engineers involved in environmental predictive fate assessments. In addition, this review is designed to be a continuing resource for environmental microbiologists interested in the areas of metabolic pathways of chlorinated aromatic compound dissimilation and biodegradative fate of these potential pollutants.

The organization of this document into specific chapters and associated review sections is intended to facilitate its use by these diverse groups of people. This is an extensive although not exhaustive review of the literature pertaining to biodegradative fate of these classes of chlorinated aromatic compounds.

SECTION 2
CONCLUSIONS

1. The biodegradability potential of a given compound depends on many factors. Chemical determinants include the ionic state of the compound, the number, types, and position of substituents, and the general form of the molecule. Environmental parameters affecting microbial metabolism include pH, temperature, redox state, moisture, reactor configuration, kinetics, and system turbulence considerations, and interference by competitive processes such as sorption, stripping, and photodegradation. Other factors involved in microbial growth and metabolism include availability of nutrients and growth factors, concentration of substrate, competitive interference by other substrates, and formation of toxic metabolites.

2. Many microorganisms require a period of acclimation before biodegradation occurs. Once a population is acclimated, it sometimes retains its predisposition to metabolize the substrate, and subsequent additions of substrate are metabolized after a shorter lag or no lag period.

3. Some compounds are mineralized by consortia of microorganisms in cases where no single species has been shown to be capable of that process.

4. Fungi and bacteria metabolize most compounds by different biochemical pathways.

5. In general, chlorophenoxy herbicides and chlorobenzenes can be metabolized to chlorophenols and then to chlorocatechols. Phenylamide herbicides and other compounds with nitrogen-containing substituents are decomposed to chloroanilines, which can be oxidized to chlorocatechols and a variety of products. Chlorocatechols may be converted by a variety of different mechanisms to nonchlorinated ring cleavage products. Chlorobenzoic acids may be transformed by three different routes to ring cleavage products: (1) through a substituted catechol, (2) through chlorosalicylic acid, or (3) anaerobically through benzoic acid to methane and carbon dioxide.

6. The above conclusions represent general pathways for substrate dissimilation. Prediction of the biodegradability of a particular compound on which no data exists, based on data about similar types of compounds, can be loosely made. For instance, the degree of chlorination affects the rate and extent of metabolism of PCBs and other compounds.

7. Experiments in the laboratory or in a particular environment cannot be readily extrapolated to other environments, because of the need to consider the parameters affecting the biodegradability potential listed above.

8. Small-scale lab tests are essential in order to perform properly controlled experiments to assess the effects of many of the above-mentioned biodegradation factors. Experimental design necessary to achieve the highly controlled results desired in small-scale testing nearly always requires compromise of factors needed to apply results to larger-scale systems.

9. Scale-up testing in larger-scale systems designed to simulate the desired full-scale application (wastewater treatment system, land farming, environmental scenario, etc.) is required to collect data (often kinetic data) allowing the prediction of process performance and comparison among full-scale systems.

10. Many of the available scaled-up studies focus on substrate disappearance or removal from the given feedstream without quantification of abiotic fates or adequate regard for calculation of biokinetic rate constants.

11. Additional emphasis should be given to establishing consistent scale-up methodology and to implementing additional work for generation of reliable scale-up data on the biological treatment of chlorinated aromatic compounds.

SECTION 3

OVERVIEW OF MICROBIAL PHYSIOLOGY

A study of the specific pathway of degradation of a compound by an organism necessarily is limited to specific biochemical reactants, products, and reactions occurring within the cell. Much of the content of this document is concerned with just such features. When genetics of the pathways are discussed, the relationship to the total cell is even more remote. Therefore, it is important to begin with a firm understanding of the physiology of the microbial cell, its structure, requirements for growth and survival, and relationship to its environment. While the cell is often described as a microscopic biochemical reactor, the activities of the cell are intimately connected to and shaped by its external environment. Information presented in this chapter is based on several general references, which may be consulted for further details (4, 107, 276).

The primary physical difference between bacteria and fungi is the presence of a membrane surrounding the DNA material. The enclosed structure is called the nucleus. Cells containing a membrane-bound nucleus are referred to as eukaryotic cells. The DNA of bacteria and cyanobacteria is contained in a diffuse region without a surrounding membrane called the nucleoid and these forms are called prokaryotes (Figure 1). Although it is tempting to consider bacteria as primitive compared to eukaryotes, the complexity of their biochemical reactions, and their regulatory and adaptive mechanisms, preclude such a label.

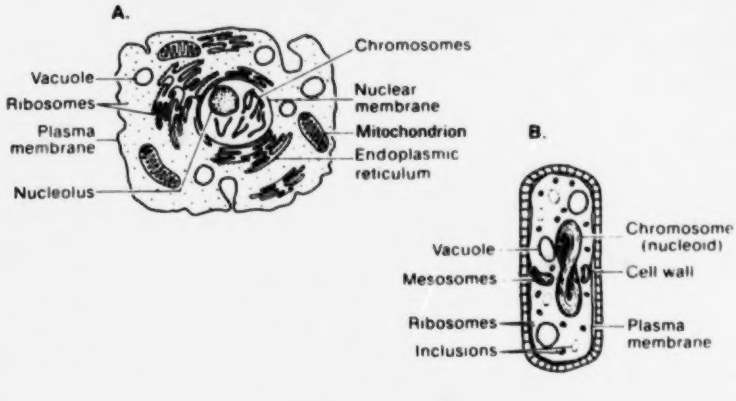

A.
Chromosomes
Vacuole
Ribosomes
Nuclear membrane
Plasma membrane
Mitochondrion
Endoplasmic reticulum
Nucleolus
B.
Chromosome (nucleoid)
Vacuole
Mesosomes
Cell wall
Ribosomes
Plasma membrane
Inclusions

Figure 1. Cellular organization. A - Typical eukaryotic animal cell. B - Typical prokaryotic rod-shaped bacterium.
Reference 4.

MICROBIAL CELL STRUCTURE

Most bacterial cells average about 1 to 2 microns in length and are rod shaped. Among all bacteria, however, cell size ranges from one tenth to 100 times the average bacteria size. Although most of the bacteria found in the environment are rod shaped, some water isolates are shaped like commas or spirals, and many bacteria, especially pathogenic species, are spherical (cocci). Other bacteria take unique shapes and forms as well.

The shape of the cell is conferred by a rigid cell wall composed mostly of peptidoglycan, lipid, lipopolysaccharide, and protein (Figure 2). There are charged polymers within the cell wall which assist in the uptake of ions and some nutrients. The wall also acts as a molecular sieve which prevents entry of some large molecules and prohibits loss of proteins, i.e., enzymes, from within the cell.

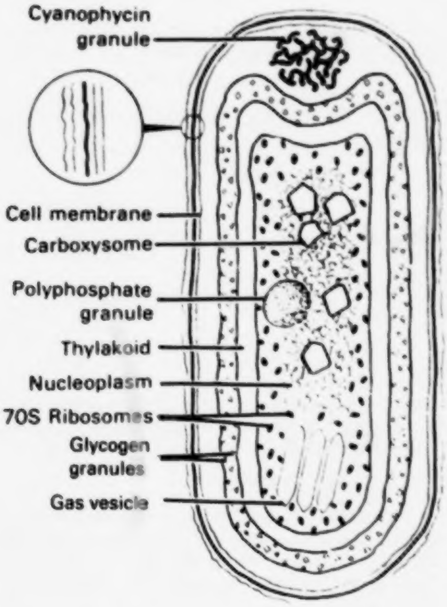

Cyanophycin granule
Cell membrane
Carboxysome
Polyphosphate granule
Thylakoid
Nucleoplasm
70S Ribosomes
Glycogen granules
Gas vesicle

Figure 2. Schematic diagram of cyanobacterial vegetative cell. (Insert) Enlarged view of cell envelope, showing outer membrane and peptidoglycan wall layers and cell membrane.

Reference unknown.

Bacteria and cyanobacteria can be divided into two groups based on cell wall structure and composition. Classically the bacteria have been differentiated into Gram positive and Gram negative groups according to a staining procedure called the Gram stain. Electron microsopic techniques have shown differences in the form of the cell walls between the two types of bacteria. The Gram positive cell wall is composed of a single dense layer of peptidoglycan. Embedded in the peptidoglycan matrix are polysaccharides and teichoic acids. The cell wall is closely associated with the cytoplasmic membrane which has a double-track appearance with a central transparent layer. The Gram negative cell wall is more complex. The outermost layer is a wavy, double-track membrane which differs in chemical composition and in function from the cytoplasmic membrane. This layer is composed of lipopolysaccharides, phospholipids, and proteins. Internal to the outer membrane is a thin rigid layer of peptidoglycan. Between the cytoplasmic membrane and the outer cell wall membrane lies the periplasmic space containing enzymes (Figure 3). In contrast, the fungi have cell walls composed mainly of polysaccharides. The particular types of polysaccharides are characteristic of the taxonomic group of the fungi.

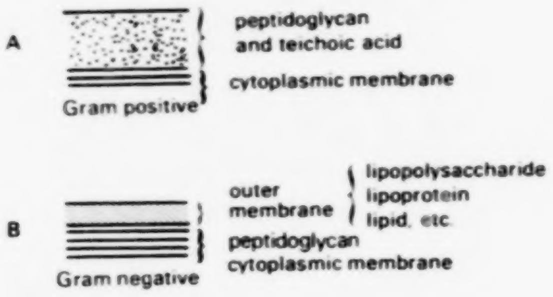


Figure 3 Cell wall structures seen in thin-section electron microscopy. A - Diagrammatic representation of the Gram-positive wall; B - of the Gram-negative wall. The location of wall components is indicated.
Reference 107.

Some bacterial cells are motile, and of these the most common mechanism is by use of flagella, hairlike helical structures several times the length of the cell. Some genera possess only 1 or 2 flagella, while in other genera the flagella are present over the entire cell surface. The flagella rotate to propel the cell through the water. Some bacteria, including some cyanobacteria, move in a characteristic gliding motion by flexing the cell wall against a surface in a manner similar to inchworm movement.

Some bacteria have the ability to attach to solid surfaces. In a few genera this may be accomplished by hairlike pili or by structures called holdfasts. In most bacteria attachment occurs by a capsule or slime layer composed of organic polymers, mostly polysaccharides. After initial contact with the surface, the cell synthesizes polymers which bridge the gap and attach firmly to the surface. It then may become impossible to remove the cell without destroying it. When the cell divides, the nonattached portion can move, but the new cell arising from the attached portion of the cell remains in place.

Other functions of the capsule or slime layer include protection of the cell from such conditions as desiccation. In many pathogenic bacteria, the presence of the capsule affords protection against white blood cells and antibodies. The capsule seems also to serve as storage sites for excess nutrients or wastes.

In freshwater environments, the cell contains a higher concentration of salts than the surrounding medium. The cell would expand and lyse without the protection against osmotic shock afforded by the cell wall together with the cell membrane. The cytoplasmic membrane is internal to the cell wall and has additional functions (Figure 4). It is selectively permeable and often facilitates movement of a substrate into or out of the cell against a concentration gradient. For other substrates transport is almost completely prevented. The rate of transport can be specific for the particular substrate, and two substrates very closely related structurally can have very different transport rates. Some substances enter or leave by passive diffusion. The cytoplasmic membrane also maintains the osmotic gradient, is the site of enzymes involved with cell wall synthesis, and is the site of oxidative metabolism and energy conversions. More complex constructions of the cytoplasmic membranes are found in specialized groups of bacteria such as the cyanobacteria and the methane-utilizing bacteria. The cyanobacteria contain internal membrane structures called thylakoids which contain the photosynthetic apparatus.

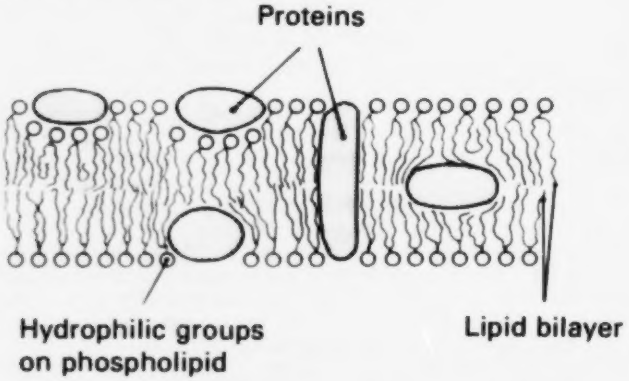


Figure 4 Structure of membranes, a diagrammatic representation. The lipid molecules are probably in constant motion.
Reference 107.

In fungi, cell growth occurs only at the tip of the hypha, and the plasma membrane below the tip contains a large number of membrane-bound vesicles which may hold the enzymes and cell wall precursors needed for cell growth. In addition, the fungal plasma membrane is involved with osmotic regulation and nutrient uptake. Functions such as oxidative metabolism are reserved to certain membrane-bound organelles which are absent in bacteria.

All cells contain chromosomal DNA; in bacteria it is circular and double stranded, resembling a helical ladder. Bacteria also contain extrachromosomal DNA called plasmids which code for auxiliary functions in the cell, such as resistance to antibiotics and heavy metals and ability to metabolize some organic compounds. Fungi contain a number of linear chromosomes. The DNA contains the code which guides the structure and metabolism of the cell. Specific features in the functioning of DNA will be discussed in a later section.

Eukaryotic cells contain mitochondria, which are organelles bounded by a double membrane. These function in ATP generation and the oxidative metabolism of substrates, activities carried out in bacteria by the cytoplasmic membrane. There are other specialized structures within some bacterial or fungal cells which function in storage of excess nutrients or gaseous products. The Gram-positive genera *Bacillus* and *Clostridium* form spores when exposed to unfavorable conditions. The spores are extremely resistant to heat, desiccation, radiation, acids, and chemical disinfectants, yet when exposed to favorable conditions will germinate and form a vegetative cell within hours.

GROWTH REQUIREMENTS

Bacteria of one type or another have been found in all environments and under all conditions with the possible exception of pure vacuum. A specific bacterial species may grow under a wide variety of conditions or it may have very exacting requirements for cell growth.

Certain nutrients are required by all cells. Carbon is most important and those cells that obtain it from organic substrates are referred to as heterotrophs. Autotrophs can fix carbon from carbon dioxide. A few specialized groups can utilize other substrates; methylotrophs, for example, can oxidize methane at aerobic/anaerobic interfaces. Other essential nutrients include phosphorus, usually derived from phosphates, and nitrogen, usually obtained from nitrate or ammonia. These three elements are the most common nutrients that limit growth. Other necessary growth factors include sulfur, magnesium, potassium, calcium, and other metallic elements. While some bacteria synthesize all their required vitamins and growth factors, other bacteria must obtain some from the environment. Water is also a specific requirement in cellular metabolism. The bacterial cell is composed of about 80% water, and water is both the solvent and a specific cofactor in many biochemical reactions.

An important physical parameter for growth is temperature. An increase in temperature may inactivate enzymes or may be lethal to the cells, while a decrease in temperature may simply inhibit growth. Cellular enzyme activity is also governed partially by the ambient temperature. Upon warming, the cells may resume normal cellular function. An individual microbial species usually has a minimum, an optimum, and a maximum temperature for growth. Those that grow best at temperatures below 20°C are called psychrophiles. Mesophiles grow from about 15°C to about 45°C, and most bacteria are grouped into this category. Thermophiles grow at temperatures above 50°C. These names permit categorization of a situation which in reality represents a gradation of microbial tolerances for temperatures ranging from the arctic environment to thermal springs.

The oxygen requirements of microorganisms vary considerably. Obligate aerobes grow only in the presence of air and use aerobic respiration to obtain energy. Obligate anaerobes grow only in the absence of air. The sensitivity of anaerobes to molecular oxygen is due to lack of enzymes which render the superoxide free radical ion harmless through reduction. Facultative anaerobes will grow in the presence or absence of air using alternate chemical electron acceptors such as O_2 or NO_3. Microaerophiles have a narrow range of tolerance for their gaseous environment and require a reduced air environment or in some cases an increased proportion of carbon dioxide.

Bacteria also respond to changes in pH of the medium. Most bacteria (neutrophiles) grow best at neutral pH (pH 7). However, acid-producing bacteria (acidophiles) grow very well at lower pH values and strains adapted to alkaline environments (basophiles) grow at pH of 8 or 9. Fungi often tolerate extremes of pH better than bacteria. Halophilic bacteria require high salt concentrations, while other species are salt-tolerant although high salt concentrations are not mandatory for survival. Organisms have been isolated from the deep ocean. Some strains survive both at the deep ocean pressure and at atmospheric pressure and are called barotolerant. Other deep ocean strains have only been kept alive by bringing them to the surface in pressurized vessels, and these bacteria are said to be barophilic. Many common strains of bacteria found at atmospheric pressure are killed when placed in a high-pressure environment.

THE CELL GROWTH CYCLE

The bacterial cell normally grows until it reaches a certain size, at which time it divides by binary fission into two identical daughter cells. During this time the bacterial DNA replicates and is partitioned to opposite sides of the growing cell. The cell wall and cytoplasmic membrane divide the cell in 1 of 2 ways depending on the particular genus.

In some genera the elongated cell pinches in equatorially until 2 cells are formed. In other genera a double cytoplasmic membrane is formed in the middle of the cell followed by synthesis of a double cell wall. When wall construction is complete the 2 daughter cells separate. Not all cells divide in synchrony, so in a culture of cells all stages of the growth cycle are represented.

Fungi grow by elongation at the tip of the hypha. Many move into a yeast stage during which the cell divides by budding. An outgrowth appears at some point on the cell surface and grows until it is almost the size of the mother cell. The cellular organelles including DNA are replicated and a copy is partitioned into the daughter cell which eventually is walled off. The mother cell does not increase greatly in size during cell division.

POPULATION GROWTH

Laboratory studies with pure cultures of bacteria traditionally have demonstrated exponential growth in batch culture, in which all essential nutrients are present in excess and growth parameters are optimal. In this situation, population growth follows a characteristic cycle which begins with the lag phase of growth, during which the cells are adapting to the new environment (Figure 5). Enzyme synthesis induced

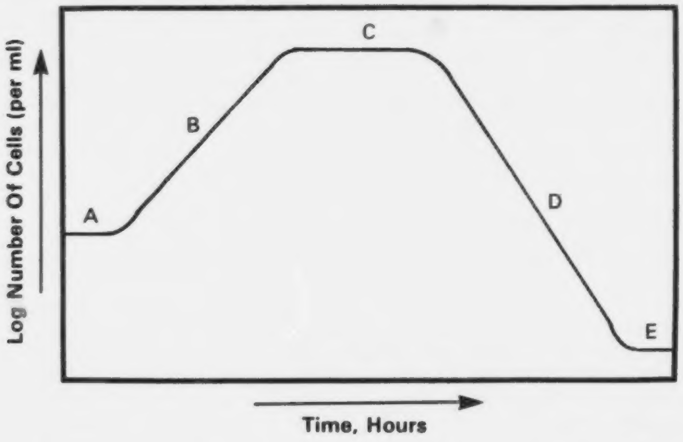


Figure 5. Bacterial growth curve. A, lag phase; B, logarithmic phase; C, stationary phase; D, decline phase; E, surviving population.

by contact with a new substrate occurs during this phase. Cells that are preadapted to the substrate or the growth conditions experience a shortened lag phase or no lag phase at all. Following the lag phase is a period of unrestricted multiplication called the log or exponential growth phase, so named because of the binary fission process of cell division. The rate of growth (cell division) depends upon the composition of the growth medium and the environmental parameters, and under optimal conditions a cell may divide every 15 minutes. When a nutrient becomes limiting or when inhibitory or toxic products accumulate, the cell enters the stationary growth phase. The individual cell is still viable although not replicating. This phase is manifested within the total culture by an equivalence between the number of cells produced by cell division and the number of cells dying; thus, there is no net change in the number of cells in the population. When the number of cells dying becomes greater than the number of cells being formed, the death phase ensues. The population eventually stabilizes at a constant low number of surviving cells. Because this cycle is characterized by an abundance of nutrients, it is rarely seen in the natural environment.

In cultures where (1) cells are able to proliferate, (2) there is an absence of inhibitors, (3) there is a homogeneous mixture of cells and nutrients, and (4) the substrate is the limiting factor in growth, cellular growth can be related mathematically with the disappearance of substrate. In this case, Monod kinetics apply as in equation 1:

$$\frac{dX}{dt} = \mu_m \frac{C_a X}{K_s + C_a} \tag{1}$$

The instantaneous change in cell concentration over time, dX/dt, is equal to the maximum specific growth rate, times a fraction including the substrate concentration in the mixed liquor, C_a, the cell concentration, X, and the Monod half-saturation constant, K_s.

The half-saturation constant is equal to the substrate concentration at which the specific growth rate is one-half μ_m (in batch culture this situation occurs at the end of the experimental growth phase. Typically the constants μ_m and K_s are determined in batch culture tests using linearized graphical plots of the reciprocals of the measured cellular growth and substrate concentrations. Alternatively, they may be determined in a series of continuous culture tests.

CONTINUOUS CULTURE

Techniques for continuous cultures have been developed to provide a constant environment for microbial growth. The physical factors of temperature, pH, O_2 concentration, etc., are well controlled, and nutrients can be supplied at controlled rates coupled with removal of potentially toxic waste materials. Population growth, therefore, occurs at a constant rate which in some systems can be varied by changing the availability of a nutrient. Studies conducted in continuous systems such as the chemostat and the recycling fermentor have helped to establish the energy requirements of cells under growth or maintenance (survival) conditions in addition to exploring the response of cells to various types of nutrient or other growth factor limitations. Maintenance of a cell population under steady-state conditions with selective pressure, such as a nonutilizable carbon source, may enable mutants with capability to metabolize the substrate to be generated and then grow to sufficient population levels to be recovered.

CELL DEATH

The most widespread measure of cell death is loss of reproductive capability. However, the medium used to detect survivors may not be adequate to demonstrate cell division, although the cell may be viable and capable of reproduction in another environment. The problem of defining cell death has not yet been resolved.

PURE AND MIXED CULTURE METABOLISM

Populations in which all the cells are of the same species are considered to be a pure culture. Except for the ongoing process of mutation, discussed in a later section, the process of cell reproduction by binary fission with replication of genetic information ensures that the pure culture will express essentially the same properties. The population will be nearly homogeneous in its ability to metabolize a substrate.

In some cases, a substrate may be metabolized only partially by a particular species and a product may accumulate. In a parallel situation, another species may be able to metabolize that product further, although the second species may lack enzymes needed to metabolize the parent substrate. By themselves, neither species could mineralize the substrate of interest. However, a mixed culture of the two organisms might act in concert with one species, mineralizing the product resulting from metabolism of the substrate by the other species. A consortium of more than two species may be required to mineralize a substrate and the effective species may be bacteria, fungi, or a mixture of the two.

Cometabolism refers to the fortuitous metabolism of a compound while the cell obtains its carbon and energy from another source (273). Such metabolism may be partial or complete and depends upon enzymes already active in the cell.

SUBSTRATE UPTAKE AND TRANSPORT

Some motile bacteria possess the ability to move along the concentration gradient of a specific compound in its environment. This phenomenon, called chemotaxis, may permit these strains to scavenge some nutrients more efficiently or to move away from toxic or inhibitory compounds.

Some bacteria are able to utilize as carbon and energy sources substrates which are too large to enter the cell. These strains secrete hydrolytic enzymes into the culture medium which break the high molecular weight compounds (such as proteins, starch, or cellulose) into smaller components which can enter the cell.

The transport of other substrates (lower molecular weight) into cells depends on a number of interrelated factors. The substrate must be able to pass the complex cytoplasmic membrane which is composed of a hydrophobic zone surrounded on both sides by hydrophilic layers. Some lipid-soluble substances can pass across this zone by free diffusion which is dependent on the difference in substrate concentration inside and outside of the membrane. The rate of uptake by this mechanism, called passive transport, depends upon the size and charge of the substrate.

Within the cytoplasmic membranes are proteins which couple substrate transport to an energy-yielding process. Called active transport, this mechanism is the route of entry for most substrates and ions, and the concentration within the cell protoplasm can be much greater than the concentration outside the cell. The proteins involved in active transport can be very specific for a particular substrate to the exclusion of structurally related analogs.

ENZYMES

Enzymes are proteins which are the most efficient known catalysts for biochemical processes. They serve to increase the rate of a reaction, often causing a reaction to occur under physiological conditions which otherwise could only occur under extremes of pH, temperature, or concentration.

Classically, enzymes were grouped into 9 categories based on function. These names are still used frequently. (1) Dehydrogenases mediate the loss of a hydrogen ion from a substrate with the acceptor being other than molecular oxygen. (2) Oxidases catalyze loss of a hydrogen ion with molecular oxygen as the acceptor. (3) Kinases transfer a phosphate group from ATP or other nucleoside triphosphate to the substrate. (4) Phosphatases mediate the hydrolytic cleavage of phosphate esters. (5) Mutases catalyze transfer of a functional group between two positions in the same molecule. (6) Synthetases mediate condensation of two separate molecules coupled with cleavage of ATP. (7) Decarboxylases achieve decarboxylation of the substrate. (8) Thiokinases catalyze the ATP-dependent formation of thiol esters. (9) Carboxylases catalyze the ATP-dependent addition of carbon dioxide to the acceptor substrate.

These categories have been replaced by 6 classes of enzymes in a formal system developed by the International Enzyme Commission (39). (1) Oxidoreductases act on the CH-OH group of a substrate, requiring NAD^+ or $NADP^+$ as the hydrogen acceptor. This category includes dehydrogenases and oxidases. (2) Transferases catalyze the transfer of an intact group of atoms, such as methyl or phosphorus containing groups from a donor to an acceptor molecule. Kinases and mutases are included in this group. (3) Hydrolases, including phosphatases, mediate the transfer of chemical groups to water. (4) Lyases, such as decarboxylases, catalyze the addition of groups to substrates containing double bonds, or the removal of groups from substrates to yield products with double bonds. (5) Isomerases catalyze a change in the atomic configuration of a molecule without a change in the number or kind of atoms. (6) Ligases are involved in the formation of a product resulting from the condensation of 2 different molecules coupled with the breaking of a pyrophosphate linkage in ATP. This class includes synthetases, thiokinases and carboxylases.

Most enzymes are notably specific in their actions, catalyzing the reaction of a particular substrate, but having no activity against a very closely structurally related substrate. Some enzymes, however, act on many related compounds. These enzymes act on a specific structural component of different substrates. Enzyme specificity is related to two features of the substrate. First is the specific chemical structure which is attacked by the enzyme. Second, the substrate must also contain a binding group which binds to the enzyme in such a way as to permit optimal association of the susceptible structure with the enzyme. The active site on the enzyme is the area containing both the binding site and the catalytic site, and the three-dimensional configuration of the complex resembles a "lock and key" relationship.

The activity of enzymes can be inhibited either irreversibly or reversibly. Irreversible inhibitors destroy or bind to a functional group on an enzyme which is necessary for its catalytic activity. Reversible inhibitors may be either competitive or noncompetitive. A competitive inhibitor has a similar structure to that of the substrate and therefore can be bound by the enzyme. However, the enzyme has no activity against the inhibitor. Since the inhibitor and the substrate compete for the binding sites of enzymes, the action of a competitive inhibitor can be partially reversed by increasing the concentration of substrate. Noncompetitive inhibitors bind to the enzyme in an area other than the binding site, and in so doing alter the catalytic site so as to make it inactive. The affected site is often called the regulatory site of the enzyme and is reversibly occupied by the inhibitor. Lowered concentrations of the inhibitor increase the activity of the enzyme. When the inhibitor is a direct product of a series of reactions involving the enzyme, the regulatory process is called feedback inhibition and is an important cellular mechanism for regulating metabolic processes such that energy is not wasted on production of unnecessary metabolites. Enzymes with a regulatory site, called allosteric enzymes, can be stimulated as well as inhibited by specific effector molecules which bind to the regulatory site. The effector molecule may be the substrate itself, signalling the enzyme to initiate the metabolic pathway. Often only one key enzyme in a pathway is regulated; the activity of the rest of the enzymes is limited by the availability of their specific substrate.

Enzymes may also be controlled at the level of enzyme synthesis. A reduction in the amount of enzyme would reduce the total enzymic activity. The genetic system for synthesis of enzymes consists of several parts (Figure 6). This general model shows several structural genes which code for the enzyme proteins. More than one enzyme may be part of a system. In addition, each system contains one control gene coding

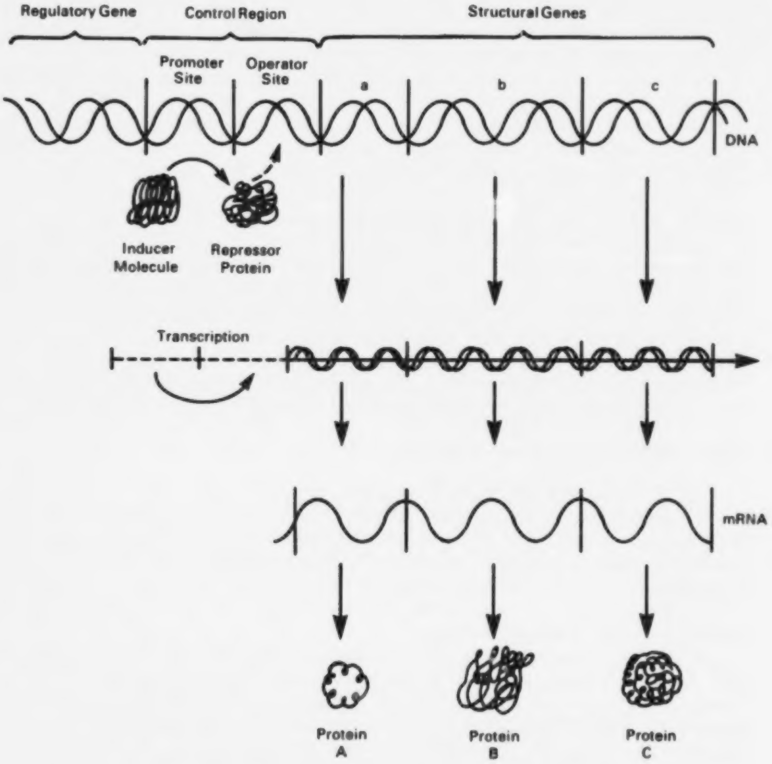

Regulatory Gene
Control Region
Structural Genes
Promoter Site
Operator Site
a
b
c
DNA
Inducer Molecule
Repressor Protein
Transcription
mRNA
Protein A
Protein B
Protein C

Figure 6. Regulation of enzyme synthesis.
Adapted from Reference 276.

for a protein, called the repressor, which binds specifically to a control site called the operator region. The repressor protein binds to the operator region in the absence of an inducer molecule, and the entire enzyme system is inactive. When the inducer is present it binds with the one repressor protein and the complex has reduced affinity for the operator region. The operator region then complexes with RNA polymerase at the adjacent promoter region and transcription of the structural genes is initiated. When the concentration of the inducer molecule falls below a critical point, the operator region is again blocked by the repressor protein and enzyme synthesis ceases.

Very long or branched metabolic pathways, in which an intermediate substrate may be directed to alternative pathways, are regulated by sequential induction, in which sections of the pathway are under separate regulatory control. The product of one series of steps acts as the inducer for the next several steps. This prevents the cell from wasting energy on unnecessary or unproductive metabolic processes.

Gene systems for a particular function which are grouped in one place physically on the genome are rare in eukaryotes. Genes for a particular metabolic pathway are more likely to be scattered over many chromosomes. However, regulatory genes still function in a similar fashion at separate control sites. Some bacteria (prokaryotes) also have systems in which the genes are scattered along the chromosome.

A more general type of control is called catabolite repression, in which the control protein binds to operator sites of many enzyme systems. This permits a favored substrate to be utilized preferentially before other substrates are metabolized. As long as the substrate of choice is present, other substrates are not metabolized, even though they may also be present. When the concentration of the favored substrate is reduced, enzyme systems for metabolism of the other substrates are induced. The favored pathway is more efficient and therefore costs the cell less energy.

Another consideration in the effectiveness of enzyme activity is the physical location of the enzyme with respect to the substrate. In eukaryotes the enzyme may be enclosed within membrane-bound organelles. In prokaryotes an enzyme may be enclosed within the periplastic space between the cell wall and the cytoplasmic membrane, while the substrate may be extracellular or intracellular.

All of the factors regulating enzyme activity may act in concert. Control of metabolic processes is finely tuned to the nutritional opportunities available, so that the cell acts in the most energy-efficient manner possible.

METABOLIC ENERGY PRODUCTION

All microorganisms need a source of energy for maintenance of cell viability and growth. The manner in which energy is obtained varies, and bacteria can be classified according to the source of their energy requirement. Phototrophs such as cyanobacteria use light directly in a photosynthetic process. Chemotrophs oxidize organic or inorganic compounds. A chemotroph which can derive its carbon requirements from carbon dioxide is a lithotroph, while a chemotroph which utilizes organic carbon is known as an organotroph.

The most common method of gaining energy is through oxidation reactions, which are normally coupled to the formation of ATP and other high energy molecules. Many different kinds of substrates can be oxidized, but eventually the substrates are modified to metabolites which can enter one of only a few pathways for carbon dissimilation. These pathways can be divided into two categories, fermentation pathways in which organic compounds serve as both the electron donor and electron acceptor, and respiration pathways in which oxygen or an inorganic compound or ion serves as the terminal electron acceptor.

Aerobes utilize a respiratory pathway known as the tricarboxylic acid cycle (Figure 7). For each mole of glucose converted to acetyl-CoA which completes the

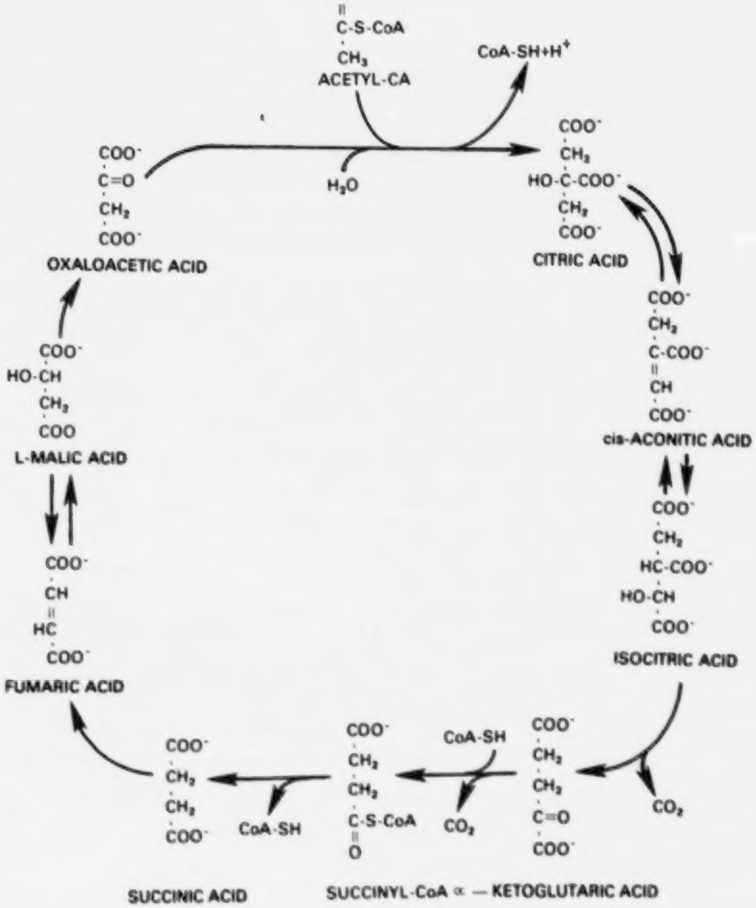

ACETYL-CA
C-S-CoA
CoA-SH+H+
H2O
OXALOACETIC ACID
CITRIC ACID
cis-ACONITIC ACID
ISOCITRIC ACID
CO2
CoA-SH
CO2
CoA-SH
SUCCINIC ACID
SUCCINYL-CoA
α — KETOGLUTARIC ACID
FUMARIC ACID
L-MALIC ACID

Figure 7. Tricarboxylic acid cycle.
Adapted from Reference 276.

cycle, 38 moles of ATP are generated. The intermediates in the cycle are precursors to important cell macromolecules and may be utilized to fulfill other needs. Other metabolic reactions act to replace the intermediates in order to maintain functioning of the cycle.

Under anaerobic conditions some facultatively anaerobic bacteria utilize anaerobic respiration. This is an oxidative process utilizing the same pathway for substrate degradation as aerobic respiration, except that nitrate or another inorganic compound is substituted for oxygen as the terminal electron acceptor.

3-KETOADIPIC ACID PATHWAY

One of the major pathways for the degradation of aromatic compounds in bacteria and fungi is the 3-ketoadipic acid pathway (Figure 8). The primary aromatic substrate is converted to either catechol or protocatechuic acid, each of which undergoes several catabolic reactions in two separate but parallel pathways, until they converge to three common intermediates, 3-ketoadipic acid enol-lactone, 3-ketoadipic acid and 3-ketoadipyl-CoA, which is cleaved to form succinic acid and acetyl-CoA. These two end products enter the tricarboxylic acid cycle. The pathway

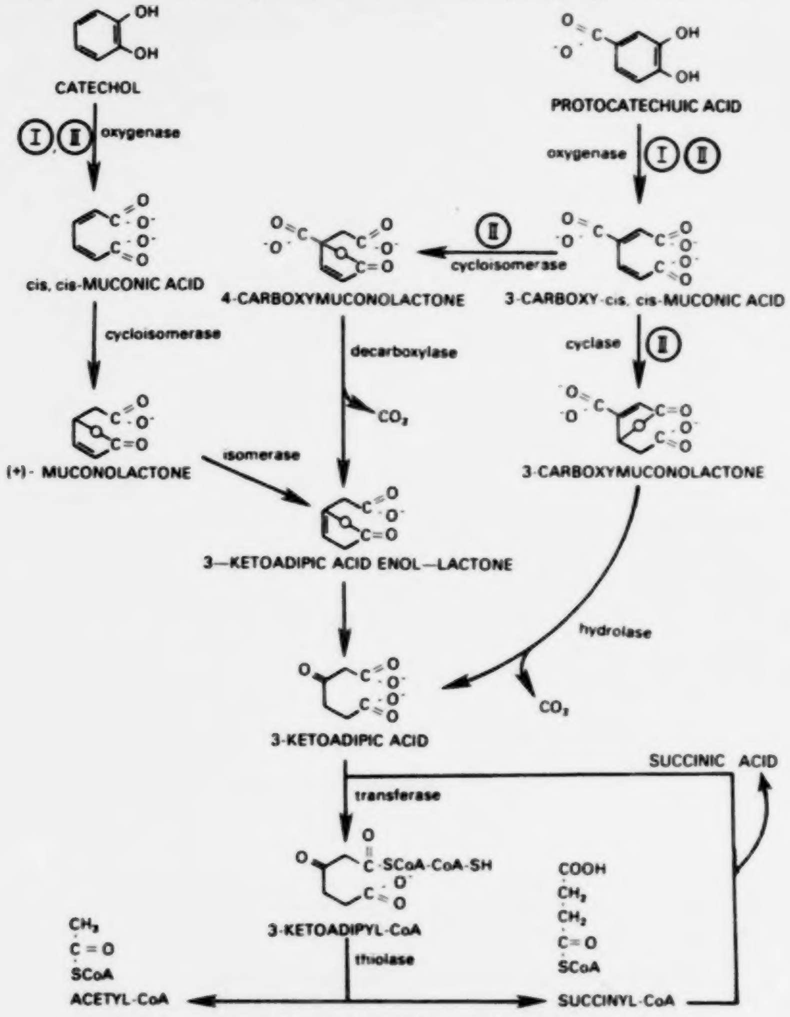

CATECHOL
PROTOCATECHUIC ACID
oxygenase
Ⓘ Ⓘⅈ
oxygenase
cis, cis-MUCONIC ACID
4-CARBOXYMUCONOLACTONE
cycloisomerase
3-CARBOXY-cis, cis-MUCONIC ACID
cycloisomerase
decarboxylase
cyclase
CO₂
(+)- MUCONOLACTONE
isomerase
3-CARBOXYMUCONOLACTONE
3—KETOADIPIC ACID ENOL—LACTONE
hydrolase
CO₂
3-KETOADIPIC ACID
SUCCINIC ACID
transferase
C-SCoA-CoA-SH
3-KETOADIPYL-CoA
thiolase
ACETYL-CoA
SUCCINYL-CoA

Figure 8. The 3-ketoadipic acid pathway in bacteria (path I) and fungi (path II). Adapted from References 66a, 93, 408.

is strictly aerobic and regulated exclusively by control of enzyme synthesis. Although the chemistry of the pathway is the same in all bacteria, the pathway is regulated differently in different groups. Details of this pathway are found in the chapter on parent compounds.

The 3-ketoadipic acid pathway is one of the best studied cellular metabolic processes. The chemistry of the pathway has been elucidated (144, 238, 289, 340, 341, 342, 409), the enzymes isolated and their amino acid composition and other properties identified (123, 307, 336, 337, 345, 348, 396, 477, 478, 479, 480), and microbial regulation of the pathway described (335, 338, 339). A comprehensive review of the pathway has been published (408).

PLASMIDS

Genes coding for vital functions of the bacterial cell are located on the chromosome and are passed to every daughter cell. However, some metabolic processes, while not essential, confer considerable advantages on cells with those capabilities. The genes for these processes are coded for on plasmids, circular strands of DNA which can replicate autonomously. Plasmids can be passed from cell tb cell as well as being replicated in the progeny; thus, an entire population can quickly acquire the specific characteristic. Traits which are often coded for on plasmids include the ability to metabolize unusual substrates including many aromatic compounds, resistance to antibiotics, and ability to survive in the presence of heavy metals. Presence of a specific plasmid is often a guide to the metabolic capability of that cell. Regulation of enzymes coded for on plasmids is similar to that discussed earlier, and catabolic repression may be effective across both plasmid and chromosomal DNA. Thus, a substrate which could be metabolized by two pathways, one on the plasmid and one on the chromosome, may be metabolized by one pathway preferentially while the other is repressed. A pathway for mineralization may involve some steps coded for on the plasmid and others coded for on the chromosome.

Since plasmids may be considered potential vehicles for genetic reassortment and transfer, they may also be viewed as mediators of evolution of biodegradative capabilities within microbial populations. At the population level, the development of DNA probes labeled with ^{32}P or fluorescent reagents can permit detection and monitoring of specific catabolic genes. Such applications would likely utilize colony hybridization techniques to directly probe for complementary target DNA in individual microbial colonies. Information derived from such experiments would allow measuring the selective pressure required to maintain catabolic genes in the natural population. In addition, the survival and transfer of novel catabolic genes originating from recombinant DNA technologies can also be tracked in the environment. Such information can be useful in the finer detailed prediction of the kinetics of biodegradation and the likelihood of utilizing genetically engineered microorganisms to degrade specific chlorinated aromatic pollutants.

SECTION 4
CELLULAR GENE CODING AND GENETIC TECHNOLOGIES

STRUCTURE AND FUNCTION OF DNA

Deoxyribonucleic acid (DNA) consists of 4 kinds of deoxyribonucleotides linked together in a specific sequence. DNA is usually double-stranded. Of the four kinds of ribonucleoside bases, there are two subsets of hydrogen bonded pairings, adenine with thymine and guanine with cytosine. RNA (ribonucleic acid) is also composed of 4 types of bases, but with uracil substituted for thymine. Each nucleoside base is joined to the carbon-1 of a pentose sugar (deoxyribose in DNA, ribose in RNA). A phosphate molecule is joined through an ester linkage to the carbon-5 and the resulting molecule is known as a nucleotide. The nucleotides are joined by linking a hydroxyl group on the carbon-3 of a pentose to the phosphate group on the carbon-5 of another pentose to form a phosphodiester bridge. The pentose carbons are primed (" ′ ") in order to distinguish them from carbons in the bases, thus the two ends of single-strand DNA are known as the 5′-phosphate end and the 3′-hydroxyl end. DNA from different species has characteristic relative amounts of the 4 nucleotides and this property has served to help identify unknown species of bacteria and establish evolutionary relationships among the species (Figure 9, Figure 10).

Double-stranded DNA consists of two strands in which the 5′-end of one strand is paired with the 3′-end of the opposite strand. The two strands are complementary — anadenine on one strand always pairs through hydrogen bonding with a thymine on the other strand, as does guanine with cytosine.

Each set of three bases along a strand is called a codon and codes for a specific message, usually formation of an amino acid. Some triplet sets of bases are stop messages while others are nonsense codons and lead to premature termination of message reading. There is some redundancy in the triplet codes. Several triplets code for the same amino acid, so a change in one base may not cause a functional change in the message. Groups of triplets code for sequences of amino acids which become proteins after some modifications. The DNA segment which codes for a single sequence of amino acids is known as a gene. The products of several genes may combine to form a protein. The total genetic material of a cell is called the genome, consisting of the chromosome and in some cases plasmids. In eukaryotic cells the chromosome includes some proteins also.

Since bases are read in groups of three, the deletion or addition of one or two bases will cause a shift in the reading frame such that the DNA is no longer read correctly. This alteration is called a frame-shift mutation and may or may not be lethal to the cell.

Hydrogen bonding as well as hydrophobic interactions of the molecules free DNA to take on a highly structured configuration resembling a double helix. Physical and chemical perturbations such as heat or acidity cause denaturation or unwinding of the DNA, eventually leading to separation of the two strands. The covalent molecules joining the bases within each strand are not broken. When the physical or

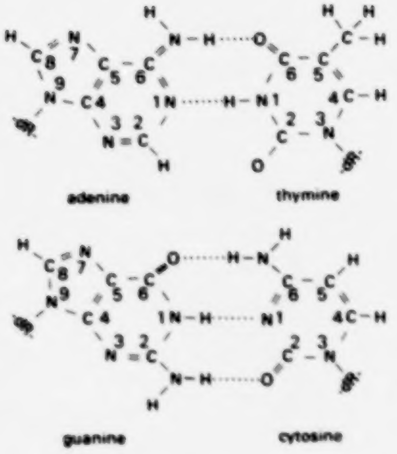

adenine
thymine
guanine
cytosine

Figure 9. The pairing of adenine with thymine and guanine with cytosine by hydrogen bonding. The symbol—dR—represents the deoxyribose moieties of the sugar-phosphate backbones of the double helix. Hydrogen bonds are shown as dotted lines.

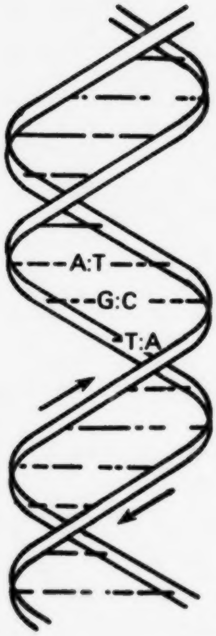


Figure 10. Schematic representation of the DNA double helix. The outer ribbons represent the two deoxyribosephosphate strands. The parallel lines between them represent the pairs of purine and pyrimidine bases held together by hydrogen bonds. Specific examples of such bonding are shown in the center section, each dot between the pairs of bases representing a single hydrogen bond. The direction of the arrows correspond to the 3′ to 5′ direction of the phosphodiester bonds between adjacent molecules of 2′deoxyribose. After J. Mandelstam and K. McQuillen, *Biochemistry of Bacterial Growth*, 2nd ed. New York: Wiley, 1973.

Reference: Stanier, K.Y., E.A. Adelberg, and J. Ingraham, *The Microbial World*, Prentice Hall, Inc., Englewood Cliffs, N.J. 1976.

chemical stress is removed, the two strands will anneal spontaneously. When denatured DNA from two different sources is mixed, a certain percentage of DNA from the separate sources will anneal to form a DNA-DNA hybrid. The amount of hybridization depends on the percentage of complementary sections between the DNA. Strands of RNA can also combine with DNA to form RNA-DNA hybrids.

The chromosomal DNA of *E. coli* consists of about 4 x 10^6 base pairs, has a molecular weight of about 2.6 x 105, and is about 1400 μm long (the *E. coli* cell is about 2 μm long). Eukaryotic cells contain from 10 to 600 times as much DNA as *E. coli* cells. Eukaryotic DNA is organized into several linear chromosomes, each carrying a unique set of genes as well as proteins.

Segments of DNA in eukaryotic cells are repeated many times, while prokaryotes usually lack repetitive sequences. Some regions of eukaryotic DNA are characterized by inverted repetitions of base sequences which may be a few to a thousand base pairs long. Eukaryotic genes also contain segments which do not code for an amino acid and are not translated. These intervening sequences or introns have been found in all eukaryotic genes yet examined. Their function is unclear. They have been postulated to contain regulatory signals or to separate the genes into smaller units which can be readily recombined into new genes.

The definition of what constitutes a gene has modified over the years as the biochemical exploration of the cell has become more detailed. A gene classically has been taken to mean the genetic material which specifies a single trait. More recently, portions of the DNA have been classified as structural genes if they code for a single polypeptide (a portion of a protein) or for a specific type of RNA, or as regulatory sequences if they function to mark the beginning and end of structural genes or start or terminate transcription.

There are two functions of the DNA molecule. The first function is to serve as a template for its own replication. Enzymes separate the two strands, add new complementary bases to each intact strand, and ligate the bases. Since the original two strands were complementary, the two new complete DNA molecules are identical, each containing one old strand and one newly synthesized complementary strand.

TRANSCRIPTION

The process of converting the information coded by DNA into RNA is called transcription and is the second function of DNA (Figure 11). Only portions of the chromosome which code for a specific sequence or sequences of required genes is transcribed at any given time. A single strand of RNA complementary to the DNA strand is generated. Most of the RNA so formed is called messenger RNA (mRNA), which codes for the amino acids which comprise the polypeptides of the proteins. Other sections generated are transfer RNAs (tRNA), ribosomal RNAs (rRNA), and regulatory sequences.

The mRNA serves to carry the genetic message from the DNA in the nucleus or nucleolus to the ribosome, a collection of proteins and RNA units which is the site of protein synthesis in the cytoplasm. A single mRNA molecule contains the message needed to code for one or several polypeptides as well as a leader region and intergenic spacer regions which are not translated.

TRANSLATION

The process of protein biosynthesis according to the code carried by the mRNA is called translation and takes place at the ribosomes in the cytoplasm. There are specific transfer RNAs that recognize each triplet codon which codes for an amino acid. The tRNA molecule has receptor sites for both the mRNA chain and specific

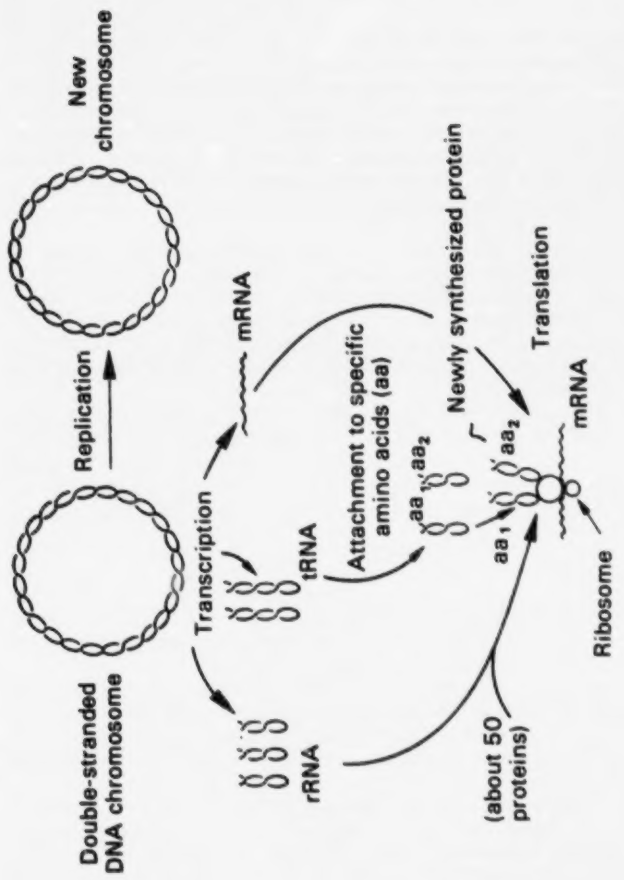

Double-stranded
DNA chromosome
Replication
New
chromosome
Transcription
mRNA
tRNA
rRNA
Attachment to specific
amino acids (aa)
aa_1
aa_2
Newly synthesized protein
Translation
mRNA
Ribosome
(about 50
proteins)

Figure 11. The general plan of synthesis of nucleic acids and proteins.
Reference: Stanier, Adelberg and Ingraham, *op. cit.*

amino acids, and serves as an adapter to bring the appropriate amino acid in close proximity to the developing polypeptide. Enzymes then attach the amino acid to the chain and remove the tRNA. When a termination codon is read, biosynthesis stops and the chain is released from the ribosome. The polypeptide finally is subjected to post-translational modification of some of its amino acids and undergoes folding into its characteristic three-dimensional shape, which renders the molecule biologically active.

Ribosomes are composed of ribosomal RNA and proteins. The rRNAs have a specific three-dimensional structure and serve as a framework for the binding of the polypeptide subunits. The ribosomal proteins are postulated to function in the process of synthesizing the polypeptide chain.

The process of translation is repetitive and a single mRNA molecule can be read simultaneously by several ribosomes spaced closely along the length of the molecule. In bacteria, translation of mRNA begins while the molecule is still being transcribed from the DNA. Thus, in prokaryotes these two processes are closely linked. The prokaryotic mRNA is quickly degraded by nucleases, so that efficient regulatory control over protein synthesis is maintained.

MUTAGENIC EVENTS

Most traits of bacteria are conservative and are reproduced in each generation. However, like all living organisms, bacteria may undergo mutations in which the genetic message is altered. If the alteration is lethal for the cell, the message is not passed on because the cell dies. Other mutations may not result in a change in expression of the message, since there is some redundancy built into DNA codes. In some cases, an alteration in the DNA code may lead to an alteration in the cell's metabolism. Some mutations allow the cell to survive at unfavorable temperatures or in the presence of potentially toxic compounds. In other cases, the cell acquires the ability to use previously unsuitable substrates. Most mutations are either lethal or place the cell at an environmental disadvantage.

Mutations occur randomly, on the order of approximately one in one million cells for a given characteristic. Some agents, including ultraviolet light, some kinds of radiation, and some chemical agents, cause increased mutagenesis. These mutations are characterized by being randomly distributed across the DNA. Mutations can also be selected by applying selective pressure to a population. For instance, in the presence of an unfavorable environment, only those cells which have mutated in such a way as to adapt to the environmental situation will survive.

Cells have powerful mechanisms for excising mutations from DNA. There are enzymes which recognize specific types of mutations and replace them with the correct message. Thus, the number of mutations passed to progeny cells may be a small fraction of the total number of mutagenic events sustained by the cell. In the case of massive mutations, such as radiation damage, the cell sets into motion a complex series of steps designed to foster cell replication at the expense of almost every other cellular function. The resultant cells are usually heavily damaged and do not function normally. The survival rate of these cells is very low.

Classical genetic techniques are based on manipulation of whole cells and environments to select and induce desired mutations. One common induction procedure is to expose a population of cells to a broadly acting mutagen and then place the surviving cells in the desired environment. Some mutations which have occurred in the genetic region of interest may enable those cells to grow or express the desired trait. A disadvantage of this method is that multiple mutations may have occurred in other genetic regions of the cell which may change the properties of the cell in unknown ways. Another method of obtaining mutations is to expose the population directly to the selective property. Only cells which can adapt will grow. However, such adaptation may take weeks, months or longer before the altered population is large enough to be observed.

Some types of genetic alterations in the cell occur as normal cellular events. Exogenous DNA can enter the cell by a variety of mechanisms and once inside can recombine with the chromosomal DNA. This process, called genetic recombination, can result in addition of new genetic information or the substitution of homologous DNA sequences. One method of genetic recombination is the transfer of exogenous DNA into a recipient bacterial cell. This process is called transformation and is the only direct evidence for DNA being the genetic material. Only a minority of recipient cells is competent at any given time to receive the DNA. Once the DNA has entered the cell, it may find its homologous region on the chromosome, recombine, and become a permanent part of the host chromosome, or it may recircularize into an autonomous plasmid which replicates and is passed to daughter cells along with the chromosome.

Conjugation is a method which permits the entry of large segments of DNA from a donor cell into a recipient (Figure 12). Direct cell-to-cell contact is required between the donor cell, which possesses a particular plasmid-encoded mating appendage through which the DNA passes, and the recipient cell which lacks the appendage. Upon contact, the donor cell is stimulated to begin replication of the plasmid and the copy is threaded through the conjugation bridge to the recipient. The donor chromosome itself can be transferred to the recipient cell if the plasmid has integrated into the chromosome. As long as contact can be maintained, transfer of genetic material continues. The transfer of genetic material always begins at the plasmid origin of replication. Therefore, by separating the cells at specific time intervals and noting which traits have been transferred, mapping of the genes along the chromosome can be achieved.

Transduction is the term given to transfer of genetic material by bacteriophages which are viruses specific for bacteria (Figure 13). During the packaging of viral DNA into phage heads in the lytic cycle, some portions of bacterial DNA will be incorporated instead. When these particles are expelled from the cell and infect another cell, the bacterial DNA is released into the new cell to recombine with homologous host DNA.

In eukaryotic cells both parental chromosomes contribute genes to the daughter chromosome. Both parental chromosomes undergo cleavage at homologous points and segments of the chromosomes are exchanged. The new combinations of genes are spliced together and passed to the progeny cells.

Some segments of DNA are highly mobile and can leave their original position in the chromosome to be inserted elsewhere. Each end of these transposable elements or transposons contains short DNA pieces called insertion sequences. The insertion sequences are recognized by specific enzymes which catalyze their insertion into new sites on the chromosome or plasmid.

In recent years techniques have been developed which permit the direct manipulation of specific genes. Many of these techniques are now well established and are being applied to solve specific problems. Descriptions of the fundamental techniques and methods follow. Additional information can be obtained from references which served as the basis for this chapter (276, 294, 334). In particular, reference 294 contains details of the methods discussed here.

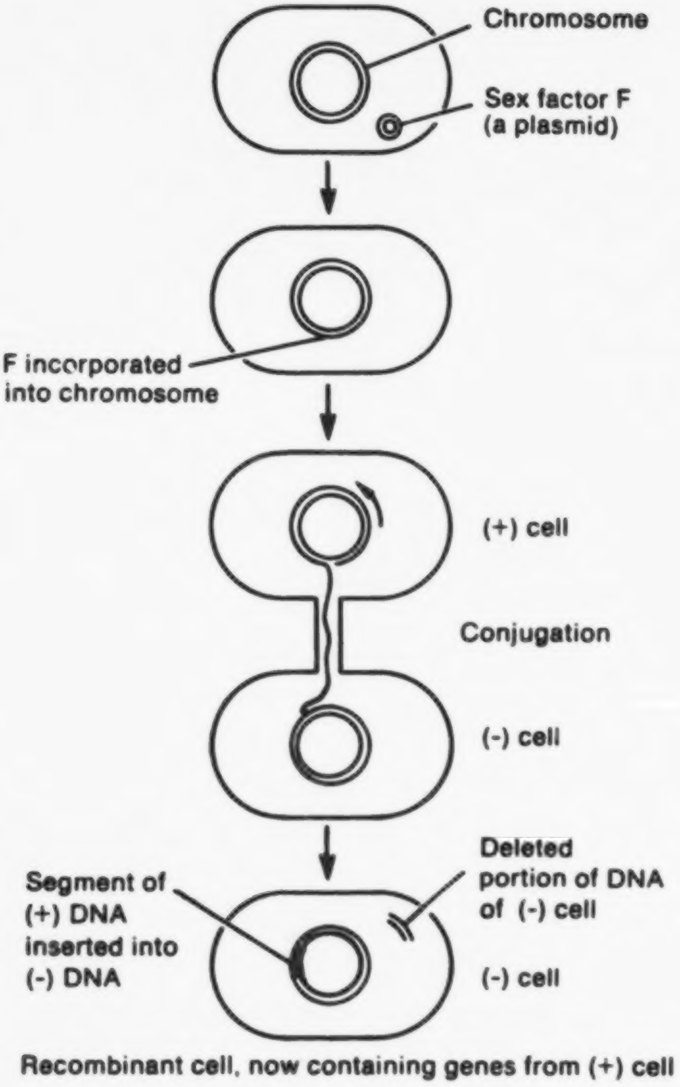

Chromosome
Sex factor F
(a plasmid)
F incorporated
into chromosome
(+) cell
Conjugation
(-) cell
Segment of
(+) DNA
inserted into
(-) DNA
Deleted
portion of DNA
of (-) cell
(-) cell
Recombinant cell, now containing genes from (+) cell

Figure 12. Transfer and recombination of genes during bacterial conjugation. The DNA of the (+) cell is replicated by the rolling-circle process, and the resulting single strand containing F is introduced into the (-) cell.

Reference 276.

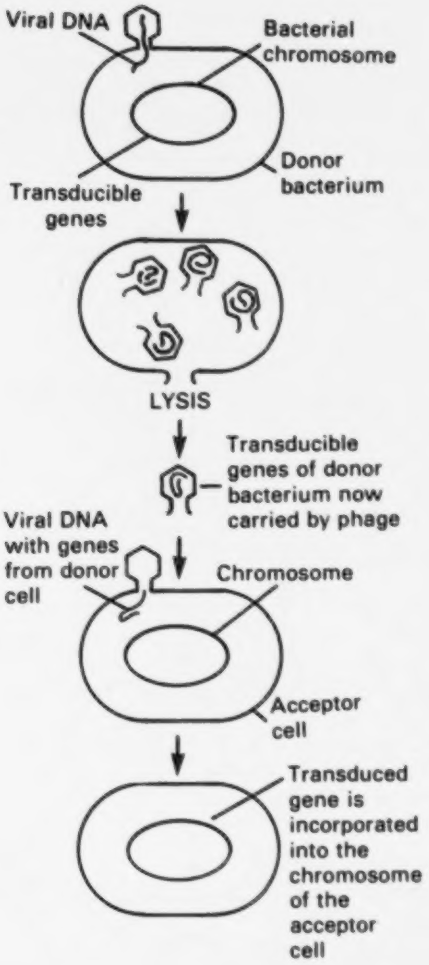

Viral DNA
Bacterial chromosome
Donor bacterium
Transducible genes
LYSIS
Transducible genes of donor bacterium now carried by phage
Viral DNA with genes from donor cell
Chromosome
Acceptor cell
Transduced gene is incorporated into the chromosome of the acceptor cell

Figure 13. Genetic recombination during viral transduction of bacterial genes into a recipient cell. Reference 276.

CURRENT BIOCHEMICAL TOOLS FOR GENETIC MANIPULATION

A series of prokaryotic enzymes has been isolated which can be used to cleave DNA and then splice different pieces together to form new strands. These enzymes are now being utilized in the laboratory.

Restriction Endonucleases

Restriction endonucleases are enzymes which specifically recognize certain sequences within double-stranded DNA. These sequences are usually 4 to 6 nucleotides long with a two-fold axis of symmetry. Examples of restriction endonuclease recognition sequences are shown in Table 1. A particular tetranucleotide recognition site might arise once in every 46 (4096) pairs, assuming random distribution of DNA base pairs. The endonucleases cleave the DNA molecules either at the axis of symmetry, yielding blunt double-stranded ends, or at positions offset from the center, giving fragments of DNA with one protruding single-stranded end known as "sticky" ends. DNA from different sources acted on by the same restriction endonuclease will produce complementary termini. In some cases, different restriction endonucleases with different recognition sequences will produce complementary termini as well. These ends can join with complementary ends on a different fragment to form new molecules.

TABLE 1. RECOGNITION SITES FOR RESTRICTION ENDONUCLEASES*

Enzyme	Recognition sequence	Termini	
	3' 5'		
Bam HI	↓ GGATCC† CCTAGG ↑	G CCTAG	GATCC G
EcoRI	↓ GAATTC CTTAAG ↑	G CTTAA	AATTC G
HaeIII	↓ GGCC CCGG ↑	GG CC	CC GG
HindIII	↓ AAGCTT TTCGAA ↑	A TTCGA	AGCTT A
MboI	↓ GATC CTAG ↑	xx xxCTAG	GATCxx xx
PstI	↓ CTGCAG GACGTC ↑	CTGCA G	G ACGTC
ThaI	↓ CGCG GCGC ↑	CG GC	CG GC

*Reference 294.

†Arrows indicate site of cleavage. (A) Adenine, (C) Cytosine, (G) Guanine, (T) Thymine.

Deoxyribonuclease

Deoxyribonuclease (DNase) is an enzyme that cleaves double-stranded or single-stranded DNA randomly, yielding fragments with 5′-phosphate termini. Depending on the conditions of the reaction, either the double strand of DNA is cleaved at approximately the same site or each strand is cleaved independently. Under certain conditions this enzyme creates nicks in double-stranded DNA which does not destroy the unity of the molecule. The concentration of DNase in the solution will affect the extent of nicking. Fragments of DNA containing regions of interest can be inserted into other molecules. Nicked regions on DNA permit insertion of nucleotides. The order of insertion can be controlled to permit creation of a defined DNA strand, and the nucleotides can be radiolabeled to permit tracking of the constructed strain during other manipulations.

Polymerases

Polymerases are enzymes that add nucleotides to the 3′-hydroxyl terminus created when the double-stranded DNA molecule is nicked by DNase. The enzyme can also remove nucleotides from the 5′-phosphate ends. Both of these processes acting at the same time result in movement of the nick along the intact strand of DNA (nick translation). If the nucleotides being added are radioactive, such as with ^{32}P, the labeled DNA can be prepared with a high specific activity. Normally the replacement nucleotides are distributed uniformly along the DNA molecule, since the nicks occur randomly throughout the DNA.

Reverse Transcriptase

This enzyme, also known as RNA-dependent DNA polymerase, uses mRNA as the template for transcription to form double-stranded DNA. Single-stranded DNA or RNA can also be utilized by this enzyme to make probes for use in hybridization experiments. Initiation of the action of reverse transcriptase requires a short DNA primer sequence base-paired to the template. Primers can be generated by exhaustively digesting DNA and retrieving the fragments. Since the fragments represent random portions of DNA, some fraction of them will bind to the template and can be used as primers. The discovery that eukaryotic mRNA contains multiple adenylate bases at the 3′-end allows construction of complementary polymer thymine residues at that site, which then will act as a primer.

The RNA template possessing a primer is then mixed with solutions of the 4 nucleotides. Usually only one nucleotide is radioactively labeled. Reverse transcriptase then catalyzes synthesis of the complementary DNA (cDNA). Following completion of the reaction, the RNA strand can be selectively degraded. Complementary DNA to be used as hybridization probes are retained in single-stranded form. However, the cDNA can form a hairpin loop at its 5′-end which acts as a self primer for synthesis of the complementary strand, or a second primer can be added to the cDNA to initiate synthesis, resulting in double-stranded cDNA.

Ligases

T4 DNA ligase links together complementary fragments of double-stranded DNA by forming a phosphodiester bond between adjacent 3′-hydroxy and 5′-phosphate ends. The ends may be either sticky (one strand of the double-stranded molecule extends beyond the other) or blunt (both strands end at the same place). T4 RNA ligase joins single-stranded RNA or DNA. These enzymes can also catalyze circularization of DNA molecules if the concentration of DNA in solution is low.

Transferases

The enzyme, terminal deoxynucleotide transferase adds deoxynucleotides to the 3′-hydroxyl end of DNA. By adding homopolymer sequences of one type of nucleotide to one set of DNA fragments, and a series of complementary homopolymer nucleotides to a second set of DNA molecules, the two populations can be joined by their newly formed complementary ends.

Methylases

These enzymes add a methyl group to particular nucleotides. Some restriction endonucleases will fail to recognize a sequence which differs only by addition of a methyl group. This is an important component of cellular defense systems, in which host DNA is methylated to protect against host restriction endonucleases which will attack foreign (nonmethylated) DNA.

MECHANICAL SHEARING OF DNA

Double-stranded DNA can be broken by the shearing forces present in solutions. Very small fragments (approximately 300 base pairs in length) can be obtained by subjecting the solution to sonication with ultrasound. Larger fragments of about 8,000 base pairs result from stirring the solution at high speed in a blender. The DNA molecule is sheared randomly along its length, producing fragments with short single-stranded ends.

CLONING VEHICLES

In order for a fragment of DNA to be replicated, it must contain a specific sequence called an origin of replication. Plasmids and prokaryotic chromosomes each usually contain one origin of replication. DNA which possesses an origin of replication is called a replicon. If a fragment of DNA in a cell cannot replicate, it will be diluted out of the population after several generations. Therefore, DNA fragments of interest must be attached to replicons, called vectors or cloning vehicles, before insertion into the cell. Replicons which are not native to the host cell may not be functional after insertion. The combination of the replicon and the foreign DNA fragment creates a hybrid molecule often called a chimera. The process of constructing a hybrid DNA molecule is known by several names, including genetic engineering or gene manipulation, to acknowledge the potential for creating new combinations of genes, and gene cloning or molecular cloning because this method allows amplification of the chimera via growth of the host population of organisms, each carrying the identical piece of genetic information. The DNA can be extracted from the new population and the chimeras recovered.

Plasmids

Plasmids are stable, extrachromosomal, circular double-stranded DNA replicons which are inheritable, but are also dispensable. Under constant selective pressure the plasmids will be replicated in the daughter cells, but when not essential for cell function many plasmids are lost from the cell. Plasmids contain from 1,000 to 200,000 base pairs. Conjugative plasmids carry a set of genes that promotes bacterial conjugation; nonconjugative plasmids lack these genes. The term relaxed plasmids refers to plasmids which are present as multiple copies (10 to 200 copies) within a single cell, while stringent plasmids are limited to 1 to 3 copies per cell. Generally, plasmids of relatively high molecular weight are conjugative and stringent, while low molecular weight plasmids are nonconjugative and are present in multiple numbers.

If cellular protein synthesis is blocked, chromosomal and stringent plasmid replication ceases, while relaxed plasmids continue to replicate and can increase their copy number to several thousand per cell. Therefore, relaxed plasmids are most useful for molecular cloning processes. Some plasmids ("promiscuous plasmids") can be transferred into a wide range of Gram-negative bacteria. These plasmids are potentially useful in transferring genetic information to diverse bacterial hosts. Some plasmids are incompatible with others and cannot coexist within the same cell. Plasmids have been grouped into incompatibility classes on the basis of mutual incompatibility.

Plasmids useful as cloning vectors generally are small and under relaxed control. They carry an easily selectable marker (such as antibiotic resistance) which allows identification of transformants which have acquired the plasmid. These plasmids also contain a single recognition sequence for a given restriction endonuclease which permits insertion of DNA into a region of the plasmid not essential for replication. A restriction site located within the marker genes will inactivate the gene when foreign DNA is inserted, providing a tracer for successful DNA insertion.

Some plasmids have been modified to include polylinkers, segments of DNA that contain closely spaced recognition sites for several restriction endonucleases. Generally, plasmids so modified are small and lack natural restriction sites. Use of small plasmids is advantageous in that they are less likely to be damaged physically during handling. Small plasmids also tend to generate higher copy numbers.

Construction of plasmid vectors involves cleaving both foreign DNA and plasmid DNA with the same restriction endonuclease to form complementary ends. Both types of DNA are mixed and are ligated. In some of the resulting molecules the foreign DNA will be ligated to the plasmid DNA, and a circular recombinant plasmid recovered. Use of a restriction site within a marker gene simplifies the process of detecting recombinant plasmids. The inserted DNA inactivates the gene. Plasmids which recircularize without insertion of foreign DNA will express the marker characteristic and can be rejected during the screening process.

Recircularization of plasmid DNA can be minimized using a procedure called directional cloning. This method takes advantage of the fact that most plasmid vectors carry single recognition sites for more than one restriction enzyme. A plasmid is digested with two such endonucleases. The larger fragment is separated and ligated with foreign DNA containing ends complementary with the two dissimilar ends generated by the two restriction enzymes. The plasmid fragment itself does not contain complementary ends and therefore will not circularize.

Another method of preventing recircularization involves treating the linear plasmid DNA with alkaline phosphatase. This enzyme removes the 5'-terminal phosphates. Ligation requires both a 3'-hydroxyl and a 5'-phosphate end. The foreign DNA combines with the treated plasmid DNA to create a circular molecule with a single nick on each strand where the phosphates have been removed. This open circular molecule can be inserted into cells much more efficiently than linear DNA and so most of the transformants will carry recombinant plasmids.

Bacteriophages

Bacteriophages (phages) are viruses which attack bacteria. The most extensively studied phage is bacteriophage l which contains a double-stranded linear DNA molecule about 50,000 base pairs long with single-stranded complementary sticky ends. The intact phage consists of the genomic material surrounded by a protein coat with a protruding tail. The tail attaches to the bacterial cell and injects only the DNA into the cell. One of two processes of replication, either lysogeny or the lytic cycle, can be initiated within the cell.

During lysogeny, the viral DNA is integrated into the host chromosome and is replicated and transmitted to progeny cells along with the host chromosome. This can happen indefinitely. At some point the lysogenic cell is triggered to begin the second process of replication, the lytic cycle. Alternatively, upon infection the viral DNA can initiate the lytic cycle immediately.

The lytic cycle begins with viral adsorption and DNA penetration. These steps require specific conditions and the phages are host specific. After entry into the host cell, the linear viral DNA circularizes via its complementary sticky ends and replicates as an independent molecule. Copies of the DNA are continuously made. Transcription of the molecule is initiated soon after replication begins. One of the earliest proteins produced is a regulatory element which acts to prevent defensive activities of the host which might otherwise prevent further transcription. During the late phase of transcription, proteins involved in assembly of the head and tail and cell lysis are produced. As many as 200 copies of a phage can be replicated within a single host cell.

During the assembly phase of the lytic cycle, a linear copy of the DNA becomes coiled into a phage prehead. When the head is filled, an additional protein attaches and locks the DNA into the head. The head finally attaches to the preassembled tail unit to form a complete phage particle. Progeny phage particles are released in a single burst when the host cell lyses. Each phage is then able to infect another cell.

Bacteriophage l Vectors

About one-third of the phage l genome is nonessential for virus replication and can be replaced by foreign DNA so that the total length of the genome is conserved. Although phage l contains several recognition sites for each of the restriction endonucleases of interest, derivatives of phage l have been developed which no longer carry restriction sites in critical areas of the genome, but carry only 1 or 2 such sites in nonessential regions. The phage l thus has been manipulated to become a useful cloning vector while still retaining its infective and lytic properties.

Cosmid Vectors

Cosmids are constructed vectors designed for cloning large fragments of eukaryotic DNA. They consist of a drug-resistance marker, a plasmid origin of replication, one or more restriction sites, and the ligated sticky end of phage l (the cos site). They are very small, so that large amounts of foreign DNA can be added to the molecule. The complete cosmid DNA is packaged into a bacteriophage coat which mediates its injection into the host cell. Inside the cell the cos site allows circularization and the plasmid origin of replication initiates replication.

Single-stranded Bacteriophage Vectors

Bacteriophages containing single-stranded DNA replicate in a different manner from phage l. After penetration, the single-stranded form is converted to double-stranded DNA which can be isolated and used as a cloning vector. The double-stranded form replicates until 100 to 200 copies are made. Then DNA replication shifts to produce large amounts of only one of the two DNA strands. Single strands are incorporated into the phage coats and the mature phage particles are continually extruded from the cell without lysing the host cell. The single-stranded DNA thus produced can be recovered and used as a template for DNA sequencing or for generating DNA probes.

METHODS OF MANIPULATING DNA

Isolation and Purification of Plasmid DNA

Plasmids inserted into cells can be amplified by growing the bacteria to high cell yields in the presence of an antibiotic providing selective pressure from the plasmid. The cells can be harvested by centrifugation and then lysed by several methods. Lysis procedures include boiling, treatment with alkali, and treatment with the surfactant sodium dodecyl sulfate. Lysozyme is added to help break apart the cell walls. The treated solution is centrifuged to remove DNA from other cellular material.

The DNA preparation contains chromosomal DNA as well as plasmid DNA. These two types of DNA can be separated by taking advantage of several differences in the properties of chromosomal and plasmid DNA. For some applications, the crude DNA preparation from as little as 10 ml of culture may be used successfully. After treatment by one of the lysis procedures, plasmid DNA is recovered from cells in intact circular form, while chromosomal DNA generally is extracted in short linear pieces. When plasmid DNA of high purity is required, centrifugation at very high speeds in a solution of cesium chloride and ethidium bromide will separate the DNA according to density. The linear chromosomal DNA takes up more of the intercalating agent ethidium bromide than the plasmid DNA. The chromosomal DNA is physically stretched more than the plasmid DNA, and therefore will be a less dense molecule. When subjected to ultracentrifugation, the two types of DNA form narrow bands in separate regions of the centrifuge tube. Contaminating protein will form a third band, and RNA will form a pellet. The ethidium bromide can be removed after this step or it can be retained during subsequent procedures.

Isolation and Purification of Bacteriophage λ DNA

Bacteriophages which are lysogenic can be recovered by inducing the bacterial culture to begin the lytic cycle. One method useful for phages containing a temperature-sensitive repressor is to raise the temperature of the culture briefly.

Phages which are not lysogenic may be amplified by infecting the host bacterial culture with a low number of phages. Much of the bacterial culture will replicate for several generations, increasing the number of host cells, before successive rounds of the lytic cycle infect the entire culture.

Cell debris remaining after completion of the lytic cycle is removed by centrifugation. The remaining solution can be subjected to density gradient ultracentrifugation, after which the intact bacteriophage particles appear as a thin band. Crude bacteriophage preparations useful for many purposes can be obtained without density gradient ultracentrifugation.

DNA can be recovered from the phage particles by treatment with a solution of a protein-digesting enzyme and sodium dodecyl sulfate. The protein components can be extracted into phenol and removed by centrifugation.

Separation and Purification of DNA Fragments

Gel electrophoresis is a sensitive method for resolving mixtures of DNA. Samples containing DNA are loaded onto a slab of agarose or polyacrylamide gel. The gel is submerged into a buffer solution of nearly the same electrical resistance and a current is applied. Various types of DNA—linear, nicked circular, and closed circular—will migrate through the gel at different rates, depending upon the molecular size of the DNA, the concentration of agarose or polyacrylamide, the applied current, and the DNA base composition and temperature. Under some conditions single strands of DNA can be separated. The DNA is stained with the fluorescent dye ethidium bromide and can be detected directly. Bands of DNA can be cut from the gel and the DNA recovered.

The DNA is then packaged into a vector and transformed into a bacterial culture for amplification. Colonies containing plasmids are grown on nitrocellulose or nylon filters seated on agar petri plates, while bacteriophage plaques are formed in a lawn of indicator bacteria on agar media and then eluted into a liquid suspension which is stable indefinitely.

Identification of Recombinant Clones

In situ hybridization of bacterial colonies or bacteriophage plaques on agar media is rapid and can efficiently screen large numbers of potential clones. Colony hybridization involves lysing the colonies on the nitrocellulose or nylon filter and then fixing the DNA to the filter *in situ*. Bacteriophage plaques are transferred to the nitrocellulose filters following plaque formation. Filters are placed in contact with plaques on the agar and then removed. Some portion of the viruses in the plaques will be removed with the filter. The DNA is then fixed, in some cases by heat treatment. The DNA probe of choice is labeled with ^{32}P and the hybridization reaction between the probe and the fixed DNA carried out.

Hybridization of Probes to Immobilized DNA

Hybridization reactions are governed by such factors as solvent used, temperature, length of hybridization, concentration and specific activity of the ^{32}P-labeled probe or density of fluorescent probe, and washing procedures after hybridization. Prior to hybridization, the filters are treated with one of a number of compounds to saturate sites on the filter with nonspecific affinity for single- or double-stranded DNA, in order to ensure that the probe DNA will not bind directly to the filter. The ^{32}P-labeled probe DNA is denatured (double-stranded molecule separated into its single-strand components) and added to the filters. During incubation, the single strands of the probe DNA will join to complementary DNA strands on the filter. Following hybridization the filters are washed thoroughly to remove unbound DNA and dried. An autoradiograph is made by placing the filters in contact with X-ray film. Following development, radioactive signals representing positive homology of the probe with plasmid or phage DNA can be correlated with colonies or plaques on the agar plates. Those colonies can be retrieved and the recombinant DNA contained therein amplified.

Other procedures employ DNA probes with induced fluorescence and/or antigenic properties for use with fluorescent antibodies to detect positive hybrids. Both methods have the sensitivity to detect as little as 1 picogram of DNA or as few as 10,000 copies of a single gene.

MAPPING OF RESTRICTION ENDONUCLEASE RECOGNITION SITES

The order of bases on a strand of DNA can be determined using a number of techniques; usually more than one is required to obtain a detailed map. By cleaving DNA with restriction endonucleases having known recognition sites, the presence and number of such sequences can be resolved.

The relative positions of dissimilar endonuclease recognition sequences can be determined by first labeling one end of linear DNA with radioactive nucleotides to obtain a reference point. Digestion of separate aliquots of the DNA by different restriction enzymes is followed by gel electrophoresis and autoradiography. Resulting fragments of different lengths define the distance of each recognition site from the labeled end, and the relative distances between each site can be determined as well.

Small fragments of DNA can be mapped using an exonuclease which digests the DNA from each end by single nucleotides. Samples of the digestion reaction are withdrawn at time intervals and are treated with restriction endonucleases. As the DNA digestion progresses, the recognition sites disappear in specific order related to their position along the molecule.

IDENTIFICATION OF DNA SEQUENCES WITHIN FRAGMENTS

The Southern transfer technique is an effective method for identifying particular sequences of DNA. Fragments of DNA created by one of the previously described methods are separated by size on agarose gels using electrophoresis. The DNA within the gel is stained with ethidium bromide and denatured (strands separated) *in situ*. The DNA is then eluted from the gel directly onto nitrocellulose filters by placing the gel on absorbent paper whose edges are trailing in a salt solution. The filter is placed on the gel and more absorbent paper placed above the filter. Wicking action will cause the DNA to migrate from the gel to the filter with the relative positions of the fragments intact. The filter is treated with ^{32}P-labeled probe DNA of known base composition and then washed well. Fragments containing complementary bands will hybridize to the probe and can be visualized after autoradiography.

EXPRESSION OF PROKARYOTIC GENES IN FOREIGN HOSTS

Most genetic engineering studies have used *E. coli* as the host with introduction of either *E. coli* genes or genes from other prokaryotes or eukaryotes. Other studies have involved introduction of genes from one strain to another strain of the same species. Little research has been conducted on expression of *E. coli* genes into other hosts. Some studies have noted that *E. coli* genes cloned into a *B. subtilis* host were not expressed, although the genes themselves when recovered and inserted back into *E. coli* were functional (334). This has been attributed to differences in the specificity of the RNA polymerases of the two hosts. There are differences as well between the translation mechanisms of *E. coli* and *B. subtilis*. Thus, *B. subtilis* genes function in *E. coli* but the reverse is not true.

GENE CLONING IN YEASTS

The yeast *Saccharomyces cerevisiae* has received the most attention with respect to application of genetic engineering techniques. This species contains a plasmid which replicates with high copy number although it has no known function (334). Fragments of yeast DNA as well as an *E. coli* plasmid vector have been cloned into this plasmid and the recombinant molecule transforms yeast with high frequency and replicates in both *E. coli* and yeast. Some yeast genes replicate autonomously and these can be used to construct vectors which transform yeasts with high efficiency.

EXPRESSION OF EUKARYOTIC GENES IN A PROKARYOTIC HOST

Genes from the fungi *S. cerevisiae* and *Neurospora crassa* have been cloned into and expressed in *E. coli* (334). However, many other genes from eukaryotic sources have been cloned into prokaryotic hosts but have not been expressed. This has been explained in part by the differences in mechanism of expression of the genes (protein synthesis). The steps involved in synthesis of a functional protein include transcription of the DNA, translation of the mRNA, and post-translational modification of the newly-formed polypeptide. Transcription and translation require a promoter or a binding site recognizable by the host RNA polymerase. Further, the protein produced is often subject to rapid degradation unless it is protected by the modified amino acids or three-dimensional configuration of the native proteins. Even if all of these components are present, genes which are expressed in a foreign host may not necessarily give rise to a stable gene product.

SECTION 5

METHODS OF BIODEGRADATION ASSESSMENT

A decade ago biodegradation was measured at a worst case level by substrate disappearance. Many reports in the area of environmental microbiology and wastewater engineering documenting biodegradation were accompanied by gas chromatographic analysis showing the net loss over time of a parent compound. In some cases, even visual disappearance of insoluble crystalline organics, such as naphthalene, was used as a gross measure of biodegradation. Most often, abiotic or sterile control samples were used in such assays of biodegradation. However, little insight was developed into nonmetabolic interactions among organisms and pollutant substrates and the measured biodegradation. In the area of wastewater treatment, BOD or COD removal measured by comparing influent and effluent concentrations was used as measures of pollutant biodegradation, with the assumption that recalcitrant organics had essentially the same fate as labile organics in wastewater treatment.

Such approaches to measuring biodegradation have been replaced by more stringent parameters to give accurate estimates of microbial catabolic potential under laboratory conditions and to determine more accurately the environmental fate of chlorinated aromatic pollutants. The most stringent criteria for accurately estimating biodegradation include mass or material balance approaches and mineralization approaches. In actual practice, both approaches are frequently integrated to give the best estimate and predictive capability of determining biodegradative fate. In either instance, the use of radiolabeled (primarily ^{14}C) substrates complements the approach, especially at environmentally realistic, low concentrations. These trace concentrations may make conventional analytical approaches more difficult and/or expensive. The approaches are summarized in Table 2.

Laboratory assays for biodegradation, using labeled or unlabeled compounds, require a determination of physical processes which contribute to the overall loss of the substrate. Accurate material balances are determined by an accounting of substrate loaded onto biomass and suspended particulates, aqueous phase substrate, residual substrate sorbed to glassware and reaction vessels, and volatilized substrate. Assuming efficient recovery for each component phase, the difference between input and accounted-for residual and comparison to abiotic control samples should give a reasonable approximation of true biodegradation. However, even under these circumstances, biodegradation may be poorly understood if aqueous phase and cellular-associated substrate is transformed to polar oxidized products. In such cases ^{14}C analysis without conventional analysis (HPLC) may underestimate biodegradation, or conventional analysis may fail to detect transformation products that in themselves are resistant to further microbial degradation. Joint conventional and ^{14}C analysis (where available) provide excellent material balance analyses for biodegradation of parent substrate and accumulated metabolic products.

TABLE 2. MATERIALS BALANCE AND MINERALIZATION APPROACHES TO BIODEGRADATION ASSESSMENT

Biodegradation approach	Process examined
Materials balances	Recovery of parent substrate Recovery of radiolabeled parent substrate and metabolic products
Mineralization	Production of carbon dioxide, methane, or their carbon radiolabeled congeners from the parent substrate Release of substituents groups, e.g. chloride or bromide ion

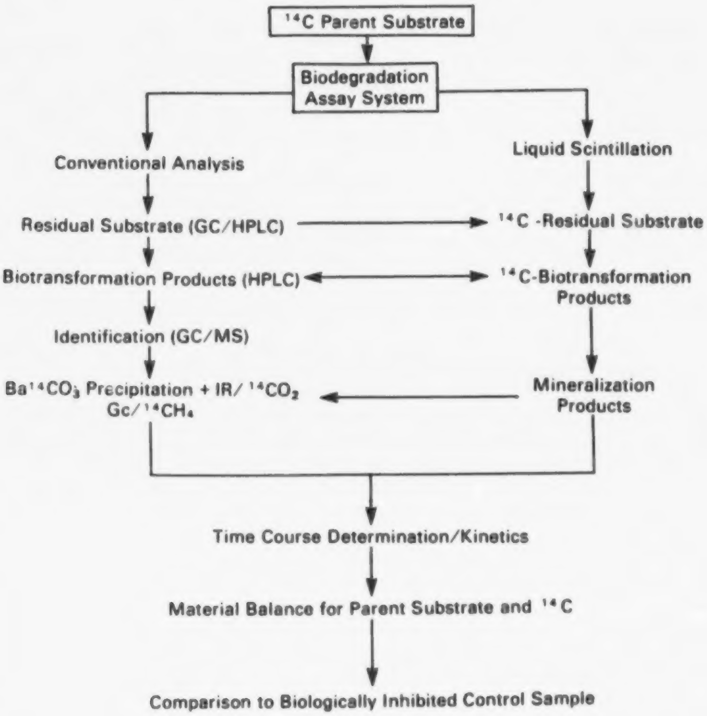
Mineralization assays as a measure of total biodegradation (oxidation or reduction to terminal decomposition products) have enjoyed utility as unambiguous measures of biodegradation. In the event that non-^{14}C-labeled substrates are employed, mineralization assays must include an absolute materials balance for the system. Such approaches have been used to study anaerobic biodegradation resulting in methane (CH_4) production as the terminal mineralization product (417).

In more general practice, CO_2 production is the most common measure of pollutant mineralization. With the commercial availability or custom synthesis of ^{14}C-labeled organic pollutants, measurements of $^{14}CO_2$ production indicating both the extent and rates of substrate degradation have become common practice.

In cases involved with biodegradation of aromatic and chlorinated aromatic pollutants, the parent substrate is generally chosen with aromatic ring-labeled ^{14}C atoms. Production of $^{14}CO_2$ during biodegradation is therefore the result of aromatic ring oxidation and cleavage, representing virtually total destruction of the parent aromatic molecule and associated bioactive properties that are of initial concern from environmental health and ecological perspectives. In alternative mineralization approaches where ^{14}C-parent substrate may be available, the release of halogen ions, generally Cl^- or Br^-, from the aromatic ring during ring oxidation and cleavage is a good measure of biodegradation. However, if the goal is to determine terminal decomposition, care must be taken to assure that halogen ion release follows ring cleavage rather than preliminary reductive or oxidative dehalogenation of the aromatic ring.

An integrated flow diagram (Figure 14) describes biodegradation assessment. Carbon-radiolabeled parent substrate is added to a reaction system containing the microbiological population of interest. In a time course fashion, samples are withdrawn or replicate samples are sacrificed. At each time point, material balances for the parent substrate and ^{14}C are prepared using a combination of conventional procedures (most conveniently analyzed by HPLC) and liquid scintillation analysis of radioactive decay of ^{14}C. Mineralized products such as $^{14}CO_2$ or $^{14}CH_4$ can be collected and analyzed by liquid scintillation analysis and confirmed by conventional analytical methods. Where specific identification and confirmation are required, mass spectrometry (MS) or GC/MS can be employed for isolated products.

The resulting data, when compared to appropriate abiotic controls to accommodate nonspecific sorption, volatilization or stripping, and photolytic or other abiotic degradation mechanisms, provide the information to determine the potential



^{14}C Parent Substrate
Biodegradation Assay System
Conventional Analysis
Liquid Scintillation
Residual Substrate (GC/HPLC)
^{14}C -Residual Substrate
Biotransformation Products (HPLC)
^{14}C-Biotransformation Products
Identification (GC/MS)
$Ba^{14}CO_3$ Precipitation + IR/ $^{14}CO_2$ Gc/ $^{14}CH_4$
Mineralization Products
Time Course Determination/Kinetics
Material Balance for Parent Substrate and ^{14}C
Comparison to Biologically Inhibited Control Sample

Figure 14. Integration of materials balance and mineralization approaches in biodegradation assessment.

and extent of degradation. A relative rank, in terms of rates of degradation, may be assigned if multiple comparisons are being made.

Biodegradation assessment may be divided into two broad categories, one comprising studies of the environment or laboratory studies which simulate the environment, and a second category which includes pure culture studies under defined environmental conditions. Environmental studies yield information on disappearance or movement of the substrate. Pure culture systems permit studies at the molecular level, including information about specific enzyme systems and gene involvement in the manifestation of degradative capability. Studies of degradation must include both mechanisms of induction and the mode of action of the enzymes. However, results from laboratory studies are not necessarily an indication of results to be expected in the environment. These studies must be correlated with environmental conditions for an assessment of *in situ* biodegradation.

Primary degradation or biotransformation is considered to be disappearance of the substrate, without consideration of metabolite formation or mineralization. While primary degradation is important evidence to assess the potential biodegradability of the molecule, only knowledge of the metabolites formed or of complete mineralization will enable confirmation of the biodegradability of the substrate under the specific environmental conditions used.

The biodegradation of a chlorinated compound is considered complete when the chloride ion is returned to its mineral state (HCl or Cl^-) and the carbon skeleton converted to cellular products (83). Appearance of the chloride ion is most conveniently measured by using an ion-selective electrode.

Disappearance of the substrate may be due to a number of factors in addition to biodegradation. These include photolytic decomposition, volatilization, chemical degradation, and sorption and irreversible binding to soils, clays, or organic matter including cells. In addition to their separate effects, these factors may work in concert with the biota to degrade the substrate. In studies of biological degradation, these factors must be controlled, eliminated, or accounted for.

CHEMICAL ANALYTICAL TECHNIQUES

The ability to quantify the amount of substrate in a given experimental system is of prime importance. To this end, sophisticated analytical techniques have been developed which allow the unambiguous identification of the substrate or its metabolites.

Gas-liquid partition chromatography (GC) is a separation technique which combines high sensitivity, accuracy, and repeatability. Low concentrations of the sample are required. The sample may be solid, liquid, or gaseous as long as the sample can be volatilized at the operating temperature of the instrument. Samples which are insufficiently volatile can sometimes be derivatized and converted to a more volatile ether or ester compound.

The sample to be analyzed is injected along with a gas which carries the sample along a column packed with inert particles coated with a liquid. The solutes in the sample are distributed between the liquid and gas phases according to the relative solubility of the solute in the liquid. Solutes of lower solubility or high volatility move through the column at faster rates. As the bands exit from the column they are recorded as roughly symmetrical peaks with retention times related to their relative partition coefficients. These can be compared with the retention times of standard materials. This does not constitute absolute proof of identity, however, as two or more solutes can elute from the column with the same retention time.

The solutes separated by GC can be analyzed directly by a mass spectrometer (MS) for determination of the molecular structures of the compounds (GC/MS). In gas chromatography-mass spectrometry, the separated sample effluent from the GC is highly ionized and accelerated in a vacuum, causing it to fragment into smaller ions. These ions are separated according to their mass-to-charge ratio (m/e) and then collected according to their relative abundance in the sample. The fragmentation pattern results from the molecular structure and is characteristic of the type of compound. The mass spectrum of the compound can be used to recreate its molecular structure and often it can be compared with mass spectra in computerized libraries for quick identification of unknown compounds.

The use of GC/MS has become widespread because of the potential unequivocal identification and quantification of metabolites and residual substrate. Materials present in minute quantities can be identified within a mixture of other materials.

Additional information regarding the types of bonds between functional groups can be obtained through infrared spectroscopy. The different types of bonds absorb at specific frequencies when infrared radiation is passed through the molecule, and the resulting spectrum is unique to the particular material. Infrared spectra are most useful when used in conjunction with other methods of compound identification and when looking for specific functional groups.

Liquid chromatography can be an effective technique for the separation of materials of either lower volatility or ionic structure. The sample, in a solvent, is passed through a column containing an absorbing material dispersed on an inert support. The solutes partition between the liquid and the absorbing material according to their relative affinities and solubilities. Each solute elutes from the column at characteristic time intervals. The great variety of both solvent and stationary phases makes this a very versatile and sensitive technique for both separation and identification of compounds by comparison with known standards. Increased resolution can be obtained by decreasing the size of the particles in the stationary phase. This formerly required high pressures to achieve the desired flow rates, leading to the term high pressure liquid chromatography (HPLC). Newer developments have reduced the required pressure and this technique is now widely referred to as high performance liquid chromatography. Solutes eluted from a liquid chromatography column can be used for other applications including mass spectrometry.

A convenient way to monitor a substrate, subjected to biodegradation testing, is to use a radiolabeled compound. When the exposure of a compound to biodegrading agents is halted, the residual quantity of the compound can be measured by counting the radioactive emission of the solution in a liquid scintillation counter. If the compound is uniformly labeled (all atoms of the given element are radioactive) then the metabolites separated by liquid chromatography can be examined for radioactivity and a determination of the fate of the original substrate can be made. This identifies the metabolites arising from the substrate and eliminates any ambiguity arising from the presence of metabolites which may have arisen from sources other than the substrate of interest.

Radioactive-carbon (^{14}C) labeled compounds are particularly useful in mineralization experiments in which carbon dioxide is evolved. Mineralization of the substrate will result in radioactive carbon dioxide which can be quantified. The advantage of this method is that it is extremely specific and gives unequivocal evidence of the extent of mineralization for the radiolabeled compound. However, few compounds are routinely available in labeled form and custom synthesis is expensive. Stringent regulations govern the use and disposal of radioactive compounds.

ANALYSES OF METABOLIC ACTIVITY

Determination of the fate of a substrate in a biological community gives specific evidence of metabolic activity. However, several nonspecific methods of analysis are available which measure the metabolic activity of the microorganisms. If the substrate of interest is the only available source of a required nutrient or energy, metabolic activity is considered to be directly related to the presence of the substrate. Lack of metabolic activity is considered to be evidence of inability of the microbial population to utilize the substrate as long as all other essential nutrients and growth factors are present. These methods do not give information about the metabolic products arising from substrate utilization.

The biochemical oxygen demand (BOD) is a measure of the oxygen required to biochemically oxidize all the carbonaceous and nitrogenous matter in a sample. This test is subject to many variations in the methods of sample collection and measurement of data, and results are comparable only between tests performed by the same protocol. The BOD test is most useful for sewage and other samples with high organic content. The test is not very sensitive as a measure of the bio-oxidation of recalcitrant substrates.

A more sensitive measurement of oxygen uptake, as a substrate is utilized, can be obtained with such manometric devices as a Warburg respirometer. The procedure is based on the ideal gas law which states that at constant temperature and constant gas volume a change in the amount of a gas can be measured by the change in its pressure. The utilization of a substrate by microorganisms usually involves utilization of oxygen and production of carbon dioxide. If the carbon dioxide is absorbed in alkali the only change in gas volume or pressure will be uptake of oxygen. Both the rate and amount of oxygen uptake can be determined by this method. Measurements may be taken at specified time intervals and a graph of oxygen uptake vs. time can be constructed. When the graph indicates a straight line function with time, the enzyme systems are usually considered to be saturated with respect to substrate, although exceptions exist and in some cases higher levels of substrate may result in an increased rate. Determination of the amount of oxygen in moles taken up per mole of substrate yields information on the completeness of substrate oxidation.

Alternatively, carbon dioxide evolution as measured by trapping in alkali gives a measure of the complete mineralization of a compound supplied as the sole source of carbon. If the substrate contains radioactively-labeled carbon, the liquid scintillation counter can be used to measure carbon dioxide evolution as captured in the alkali trap.

Warburg respirometry has been used to develop the technique of simultaneous adaptation (407) for determination of the involvement of specific compounds in the pathway of substrate metabolism. Simultaneous adaptation is based on the theory that cells adapted to metabolize the primary substrate will also be adapted to metabolize all the intermediates of the pathway, but will not attack other substrates. In respirometry tests, this is seen as immediate uptake of oxygen after addition of the substrate or its metabolites. When other substrates are introduced, no uptake or uptake only after a lag period is observed. The only prerequisite for this system is that the enzymes be largely adaptive, such that they are not induced until the specific substrate is present. Therefore, a limitation to this test is involvement of nonspecific enzymes.

Assays have been developed for determining the presence of specific enzyme in a solution or culture. The assays are usually designed so that a substrate which is acted on directly and specifically by the enzyme of interest is made available. Enzyme activity is monitored by measuring loss of the substrate or appearance of a product by spectrophotometric, chemical or chromatographic methods. These assays are most useful for enzymes which are specific for a single substrate. Some enzyme assays require that a cell-free culture filtrate be prepared, while others work with intact cells.

Enrichment cultures are widely used to search for organisms with degradative ability. The substrate of interest is supplied as the growth-limiting nutrient in a culture medium to which a mixed culture of microorganisms is added. The substrate most commonly is the sole source of carbon, and the inoculum may include sewage sludge, sediment samples, or river or ocean water. An inoculum is often selected from environments thought to be contaminated with the substrate of interest. Only organisms with ability to degrade the substrate will be able to grow in the culture medium and eventually will become the dominant population. These cells then can be recovered and isolated from the other cells added in the original inoculum. Evidence of substrate utilization is obtained indirectly by measuring growth of the population of the degradative organism, indicating incorporation of the substrate into cellular material. Further tests, usually with the isolated culture, are required to determine if the substrate is completely mineralized or whether intermediate metabolites remain. As some bacteria can grow in the absence of specifically added carbon, control experiments must be performed to ensure that the substrate is necessary for growth.

PARAMETERS FOR PURE CULTURE STUDIES

Bacteria used for pure culture studies may be selected from enrichment cultures. Identification of these isolates permits the results of such studies to be analyzed in the context of other such studies. Investigations of specific enzymatic or genetic features of degradative bacteria are more easily integrated with other studies when reference bacteria are used. These reference bacteria represent isolates which have been identified and then deposited in culture libraries such as the American Type Culture Collection (ATCC) or the National Collection of Industrial Bacteria (NCIB). Bacteria registered with such libraries are always indicated by a reference number which permits other researchers to obtain the same strain for subsequent studies. However, bacteria frequently mutate during repeated subculturing in a laboratory, and a strain studied for a length of time may no longer resemble the original culture. For this reason, the conditions under which a strain is maintained should be reported. Of particular importance is the frequency with which bacteria lose their degradative capability when removed from the substrate of interest. Such cultures must be maintained on media containing the substrate.

Some general parameters for biodegradation in solution are:

- The concentration of the substrate is an important consideration in all studies of biodegradation. The capability of bacteria to degrade substrates supplied at trace levels may be very different from the response to high concentrations.
- Chemicals used in formulating culture media should be of the highest purity possible, particularly when the contaminating chemical may be implicated in the degradative strategy of the organism.
- Parameters such as pH, temperature, and dissolved oxygen should be monitored, as fluctuations may affect not only the metabolic activities of the bacteria but also the chemical nature of the substrate.
- As newer techniques of analysis become available, broad studies comprising many of the techniques discussed here become feasible. Knowledge of both the environmental degradative behavior of the bacterial culture and its genetic structure can lead to manipulation of the culture toward increased degradative capability.

SECTION 6

METABOLISM OF NONCHLORINATED AROMATIC COMPOUNDS

CHEMISTRY OF BENZENE AND SUBSTITUTED BENZENES

The basic chemical structure of most aromatic compounds is the benzene ring. Benzene (molecular formula C6H6) is the simplest six-membered aromatic carbocycle. The entire molecule is structurally flat, i.e., the six carbon atoms and attached hydrogens lie in the same plane. The six aromatic electrons are delocalized and thereby confer the stability which is inherent to aromatic structures. The stability of benzene refers to the availability of the aromatic electrons for bonding. When the reactivities of aromatic and aliphatic carbocycles are compared under identical conditions, the aromatic systems are found to be less reactive, hence more stable.

The naming of substituted benzenes can be quite confusing due to several completely different sets of nomenclature rules. For instance, there is a set of trivial names such as toluene, phenol, and aniline. These trivial names are enigmatic to the casual observer since there are no rules, only a historical selection. Positional isomers have at least two nomenclature systems. The terms *ortho, meta,* and *para* have been used to identify the position of the substituents attached to the benzene ring. Finally, the International Union for Pure and Applied Chemistry offers a numerical description for positional isomers (Figure 15). To show the interrelationships of these systems, catechol, for example, is the trivial name for 1,2-dihydroxy benzene and a more cumbersome *ortho*-hydroxy phenol.

Positional location of substituents *(ortho, meta, para* or 1,2; 1,3; 1,4) is important to the overall reactivity of an aromatic molecule. A substituent on the benzene ring substantially influences the mode of reaction for a given chemical or biochemical system, i.e., the where and how of the attack by another reactive molecule. Common substituents attached to chlorinated aromatic molecules, available common articles of commerce, are: the hydroxyl (-OH), amino ($-NH_2$), methyl ($-CH_3$), and phenyl ($-C_6H_5$) groups which can render the molecule more reactive to electrophilic ("electron-loving") reaction conditions. Additional halogen substituents serve to deactivate the aromatic ring for electrophilic attack. Each of the cited substituent groups has a directing influence on subsequent electrophilic substitution which occurs mainly at the *ortho* and *para* positions (316).

The chemical nature and structural position of a substituent on the benzene ring affects the mode and ease of microbial attack on the compounds. The biological "recalcitrance" of a molecule, i.e., the resistance of a molecule to microbial degradation, is a direct consequence of the chemical nature and structural position of a substituent on the aromatic ring (2). Mention throughout this work of the recalcitrance of a compound will refer to biological activity rather than chemical or photooxidative processes.

MICROBIAL ATTACK ON BENZENE STRUCTURES

The first step in oxidative microbial attack on benzene involves hydroxylation of the ring. This is accomplished by different mechanisms in prokaryotes (bacteria) and

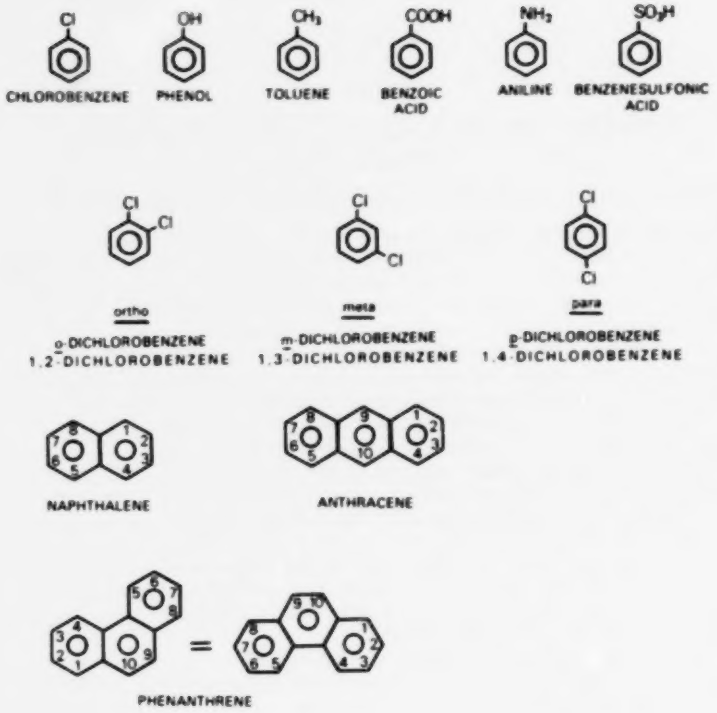

Cl
OH
CH₃
COOH
NH₂
SO₃H
CHLOROBENZENE
PHENOL
TOLUENE
BENZOIC ACID
ANILINE
BENZENESULFONIC ACID
Cl
Cl
ortho
o-DICHLOROBENZENE
1,2-DICHLOROBENZENE
meta
m-DICHLOROBENZENE
1,3-DICHLOROBENZENE
para
p-DICHLOROBENZENE
1,4-DICHLOROBENZENE
NAPHTHALENE
ANTHRACENE
PHENANTHRENE

Figure 15. Common names and conventional nomenclature for substituted benzenes.

eukaryotes (fungi, mammals, etc.). Bacteria employ a dioxygenase which incorporates two atoms of oxygen from an oxygen molecule simultaneously into the ring (Figure 16) (15, 175, 205). Molecular oxygen is a required substrate for this enzyme (84). The resultant intermediate compound is a *cis*-1,2-dihydroxy-1,2-dihydrobenzene which loses two hydrogens to become catechol (1,2-dihydroxybenzene), a process mediated by the enzyme *cis*-benzene glycol dehydrogenase (14). The stereospecific nature ("*cis*") of the intermediate compound was identified in 1968 (181) and confirmed in 1970 (178) for *Pseudomonas putida*, and since then has been shown to be true for all bacterial species studied (14, 176, 177, 205).

Substituted benzenes similarly are oxidized to *cis*-dihydrodiols (substituted catechols) by bacteria. Some species oxidize the substituent before hydroxylation of the aromatic ring while others attack the ring yielding a substituted catechol. For example, toluene is attacked by *P. aeruginosa* with oxidation of the methyl group to benzyl alcohol, benzaldehyde and benzoic acid, followed by ring hydroxylation to form catechol (254, 331), while *Achromobacter* spp. and *Pseudomonas* spp., including *P. putida*, hydroxylate toluene directly to form 3-methylcatechol (Figure 17) (82, 175, 176, 179, 182, 332). Other alkylbenzenes are also subject to these two types of oxidative degradation, either oxidation of the aromatic ring to form an alkylcatechol or oxidation of the alkyl substituent to form an aromatic carboxylic acid which is dihydroxylated to catechol (84, 177, 177a, 181).

Benzoic acid is metabolized by a number of different pathways, depending on the bacterial strain. *Alcaligenes eutrophus* oxidizes benzoic acid to catechol. This mechanism in *A. eutrophus* is by way of the 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid intermediate (74, 368) catalyzed by benzoic acid 1,2-dioxygenase, a two-protein enzyme composed of NADH-cytochrome *c* reductase and another protein (475). The intermediate is converted to catechol by a single protein (Figure 18). The fluorescent pseudomonad group also oxidizes benzoic acid to catechol (410, 467). In contrast, the acidovorans pseudomonad group monohydroxylates benzoic acid to *m*-hydroxybenzoic acid and subsequently to either gentisic acid *(P. acidovorans)* by *m*-hydroxybenzoic acid 6-hydroxylase or protocatechuic acid *(P. testosteroni)* by *m*-hydroxybenzoic acid 4-hydroxylase (467). Other *Pseudomonas* spp. monohydroxylate benzoic acid to *p*-hydroxybenzoic acid by utilizing benzoate 4-hydroxylase, and metabolize this intermediate further to protocatechuic acid by the enzyme *p*-hydroxybenzoate 3-hydroxylase (Figure 9) (82).

Pseudomonas PN-1 metabolizes benzoic acid, *p*-hydroxybenzoic acid and *m*-hydroxybenzoic acid to protocatechuic acid aerobically. However, under anaerobic conditions of nitrate respiration both protocatechuic acid and *m*-hydroxybenzoic acid are metabolized through *p*-hydroxybenzoic acid to benzoic acid. The mode of attack resulting in ring cleavage has not been elucidated (430, 431).

Members of *Streptomyces* spp. metabolize benzoic acid via catechol, *m*-hydroxybenzoic acid via gentisic acid, and *p*-hydroxybenzoic acid via protocatechuic acid (421). Two pathways have been shown in facultatively thermophilic *Bacillus* spp. In one species, benzoic acid, *m*-hydroxybenzoic acid and *p*-hydroxybenzoic acid are all metabolized via gentisic acid (64). The conversion of *p*-hydroxybenzoic acid to gentisic acid by this organism requires an "NIH Shift" of the carboxyl group. In another species, benzoic acid is metabolized through salicylic acid to 2,3-dihydroxybenzoic acid (406). The latter pathway is analogous to that shown for *Azotobacter* sp. (450).

The phototrophic bacterium *Rhodopseudomonas palustris* is unable to use benzoic acid as a substrate for aerobic growth (191), although early work postulated an aerobic pathway via protocatechuic acid and catechol (357). The organism can metabolize *p*-hydroxybenzoic acid aerobically via the protocatechuic acid path (125). Under anaerobic photosynthetic conditions, however, benzoic acid is

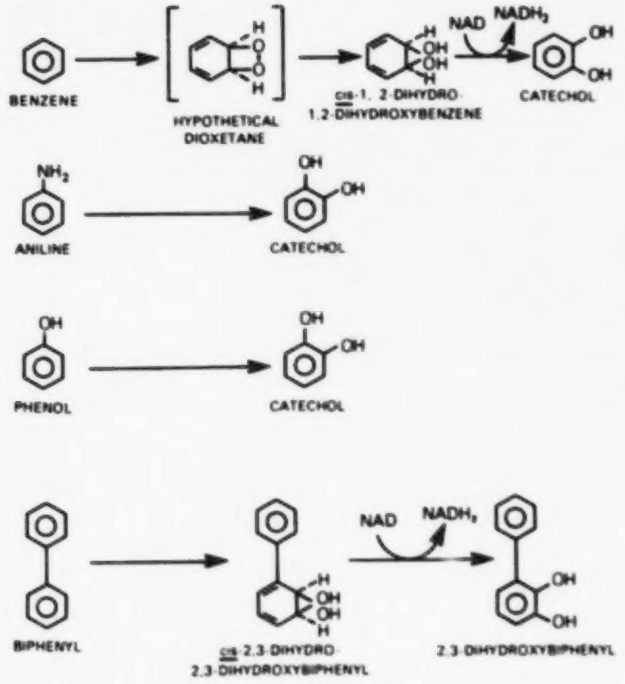

BENZENE
H
O
O
H
HYPOTHETICAL DIOXETANE
H
OH
OH
H
cis-1, 2-DIHYDRO-1,2-DIHYDROXYBENZENE
NAD
NADH₂
OH
OH
CATECHOL
NH₂
ANILINE
OH
OH
CATECHOL
OH
PHENOL
OH
OH
CATECHOL
BIPHENYL
H
OH
OH
H
cis-2,3-DIHYDRO-2,3-DIHYDROXYBIPHENYL
NAD
NADH₂
OH
OH
2,3-DIHYDROXYBIPHENYL

Figure 16. Oxidation of aromatic molecules by bacteria.
Adapted from References 14, 16, 63, 71a, 141, 176, 178, 183, 196, 287, 326.

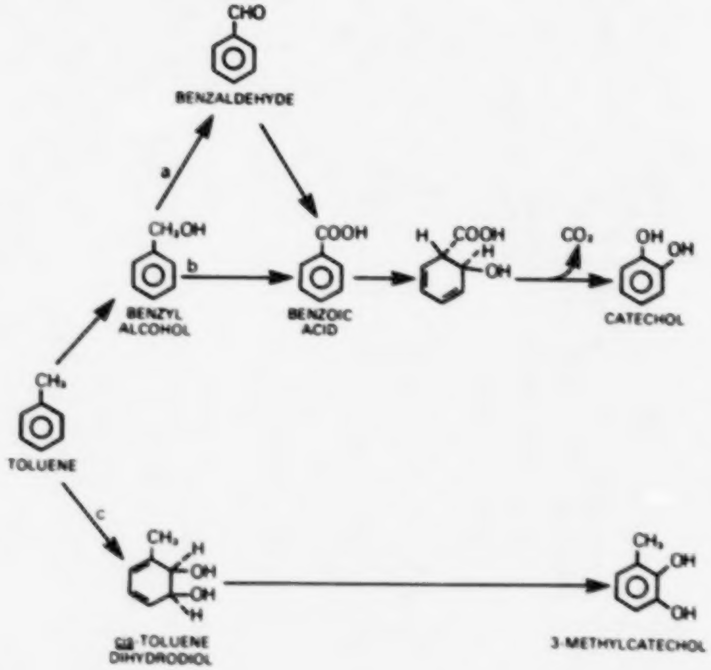

CHO
BENZALDEHYDE
a
CH_2OH
b
BENZYL ALCOHOL
$COOH$
BENZOIC ACID
H
$COOH$
H
OH
CO_2
OH
OH
CATECHOL
CH_3
TOLUENE
c
CH_3
H
OH
OH
H
cis-TOLUENE DIHYDRODIOL
CH_3
OH
OH
3-METHYLCATECHOL

Figure 17 Pathways for the bacterial metabolism of toluene. Pathway (a)*P. putida* mt-2; (b) *P. aeruginosa*; (c) *P. putida*; *Pseudomonas* sp.; *Achromobacter* sp. Adapted from References (a) 471a; (b) 254; (c) 92, 179.

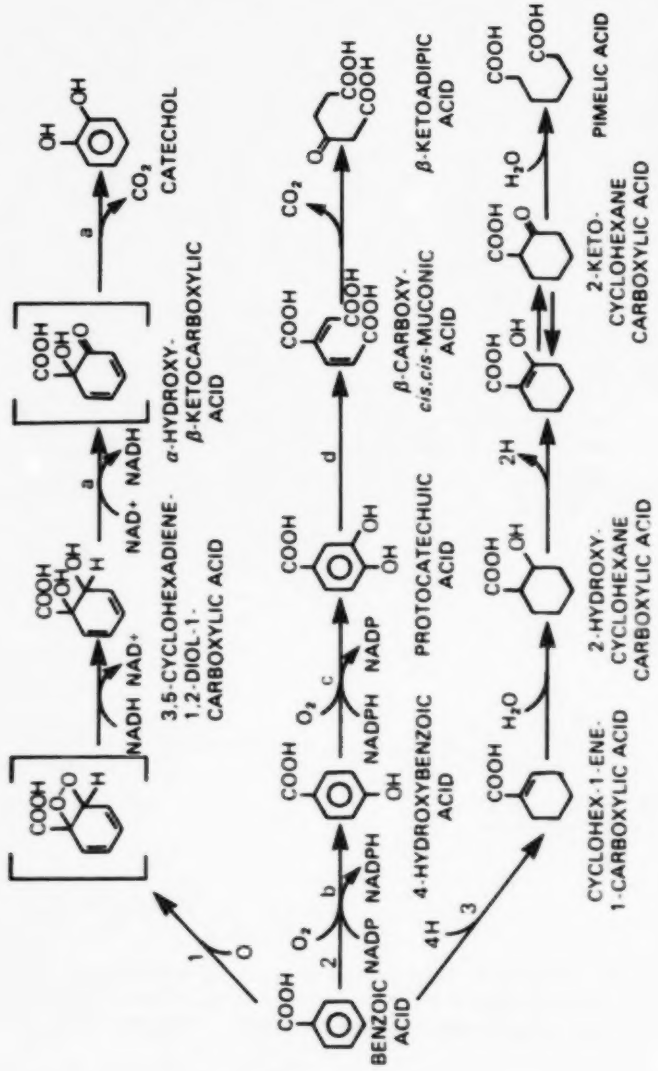

COOH
BENZOIC ACID
1
O
2
O_2
NADP
b
NADPH
4H
3
4-HYDROXYBENZOIC ACID
O_2
c
NADPH
NADP
PROTOCATECHUIC ACID
d
β-CARBOXY-cis,cis-MUCONIC ACID
CO_2
β-KETOADIPIC ACID
CYCLOHEX-1-ENE-1-CARBOXYLIC ACID
H_2O
2-HYDROXY-CYCLOHEXANE CARBOXYLIC ACID
2H
2-KETO-CYCLOHEXANE CARBOXYLIC ACID
H_2O
PIMELIC ACID
NADH NAD+
3,5-CYCLOHEXADIENE-1,2-DIOL-1-CARBOXYLIC ACID
a
NAD+ NADH
α-HYDROXY-β-KETOCARBOXYLIC ACID
a
CO_2
CATECHOL

Figure 18. Pathways for the metabolism of benzoic acid. (1) *A. eutrophus*; (2) soil pseudomonad; (3) *R. palustris* (anaerobic photometabolism). Enzymes: (a) 3,5-cyclohexadiene-1,2-diol-1-carboxylate dehydrogenase; (b) benzoate 4-hydroxylase; (c) *p*-hydroxybenzoate 3-hydroxylase; (d) protocatechuate oxygenase.

Adapted from References (1)368; (2)82; (3)124, 125, 191.

metabolized reductively to pimelic acid via cyclohexanol (Figure 18) (124, 125, 191). Early decarboxylation does not occur. The enzymes involved are thought to be reductases such as ferredoxin coupled to the light-induced electron transport system (125).

Rhodococcus sp. strain AN-117 utilizes aniline as a sole source of carbon and energy and metabolizes it exclusively by conversion to and *ortho* cleavage of catechol by inducible enzymes (235). In contrast, strain SB3, thought to be a pseudomonad, utilizes a constitutive *meta* cleavage pyrocatechase and hydroxymuconic semialdehyde dehydrogenase. However, aniline degradation occurs only when the cells are grown on aniline, indicating the presence of another inducible enzyme. Aniline-grown resting cells of *Frateuria* sp. ANA-18 oxidize aniline without a lag and oxidize catechol at a faster rate than aniline (9). Metabolites resulting from incubation of a cell-free extract with aniline include *cis, cis*-muconic acid, beta-ketoadipic acid and ammonia.

A mutant strain of a *Nocardia* sp. has been shown to convert aniline to catechol via simultaneous dioxygenation (16). This pathway is partially corroborated by data indicating that a *Pseudomonas* sp. grown on aniline oxidizes catechol rapidly, 4-aminophenol moderately quickly, 2-aminophenol slowly, and phenol not at all (449). However, only half the ammonia theoretically expected from the direct formation of catechol from aniline was recovered. This discrepancy has not been resolved. *P. multivorans* strain AN1 growing on aniline was simultaneously adapted to oxidize catechol but not phenol or 2-aminophenol (199). Transient formation of a catechol was noted indicating replacement of the amine group with a hydroxyl.

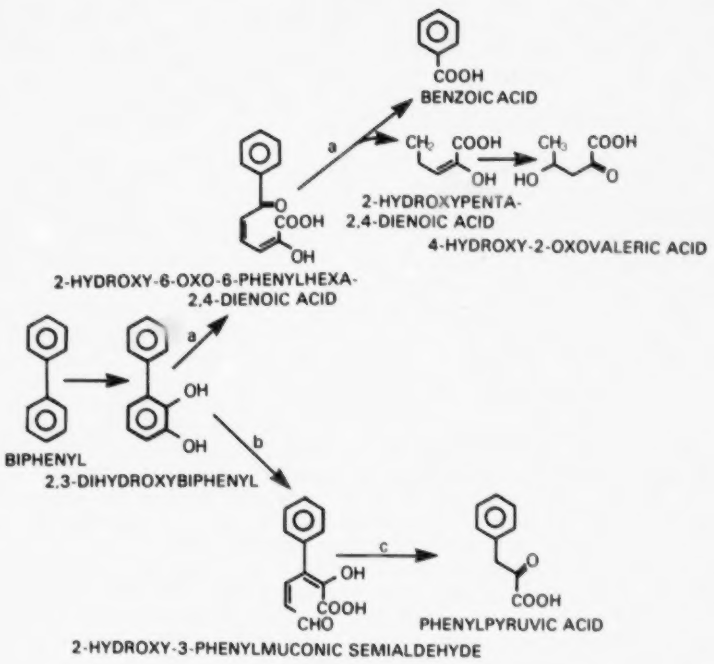
Biphenyl (a benzene-substituted benzene) is dihydroxylated by *Beijerinckia* sp., *P. putida*, and an unidentified Gram-negative bacterium to *cis*-2,3-dihydro-2,3-dihydroxybiphenyl and subsequently to 2,3-dihydroxybiphenyl (Figure 19) (183, 287). Phenol is oxidized directly to catechol via phenol hydroxylase (63, 141, 196, 326).

Polynuclear aromatic hydrocarbons are made up of fused aromatic rings. The three simplest compounds, naphthalene, anthracene, and phenanthrene, are metabolized by bacteria to form *cis*-dihydrodiols by the same mechanism as that shown for benzene (Figure 20) (72, 227). A mutant strain of *Beijerinckia* sp. (strain B836), as well as *P. putida* strain 199, forms (+)-*cis*-1,2,-dihydroxy-1,2-dihydroanthracene from anthracene (226). These two organisms also convert phenanthrene to (+)-*cis*-3,4,-dihydroxy-3,4-dihydrophenanthrene. A minor product formed is *cis*-1,2-dihydroxy-1,2-dihydrophenanthrene and there were no other diols recovered during these experiments. Pseudomonads also oxidize phenanthrene to 3,4-dihydroxyphenanthrene (Figure 21) and then to 1,2-dihydroxynaphthalene which is metabolized via the naphthalene pathway (Figure 22) (145, 267, 282, 373).

Naphthalene is metabolized by pseudomonads through *cis*-1,2-dihydro-1,2-dihydroxynaphthalene, 1,2-dihydroxynaphthalene, salicylaldehyde, salicylic acid, and catechol (106). The enzyme which catalyzes the conversion of the 1,2-dihydro-1,2-dihydroxynaphthalene to 1,2-dihydroxynaphthalene is *cis*-naphthalenedihydrodiol dehydrogenase (224). Its proposed mechanism is stepwise, the first step bacterial-enzyme catalyzed and the second step a nonenzymatic enolization (224).

Anthracene is oxidized to 1,2-dihydroxyanthracene by a naphthalene-grown *Pseudomonas* sp. (151, 374). This intermediate is metabolized further to 2,3-dihydroxynaphthalene which follows an unknown degradative pathway through salicylic acid (Figure 23) (145).

In all of the above examples with the exception of phenol, bacterial attack on the benzene ring proceeds via a dioxygenase with formation of a *cis*-dihydroxybenzene. This mechanism is consistent with almost all bacterial oxidations of all substituted benzenes studied to date. The phenol hydroxylase is a monooxygenase but results in an *ortho*-dihydroxylated molecule.



COOH
BENZOIC ACID
a
CH2
COOH
OH
CH3
COOH
HO
O
2-HYDROXYPENTA-2,4-DIENOIC ACID
4-HYDROXY-2-OXOVALERIC ACID
O
COOH
OH
2-HYDROXY-6-OXO-6-PHENYLHEXA-2,4-DIENOIC ACID
a
OH
OH
BIPHENYL
2,3-DIHYDROXYBIPHENYL
b
c
OH
COOH
CHO
O
COOH
PHENYLPYRUVIC ACID
2-HYDROXY-3-PHENYLMUCONIC SEMIALDEHYDE

Figure 19. Metabolism of biphenyl by (a) *P. putida* and (b) *Beijerinckia* sp. Adapted from References (a)70, 71; (b)183, 287; (c)287.

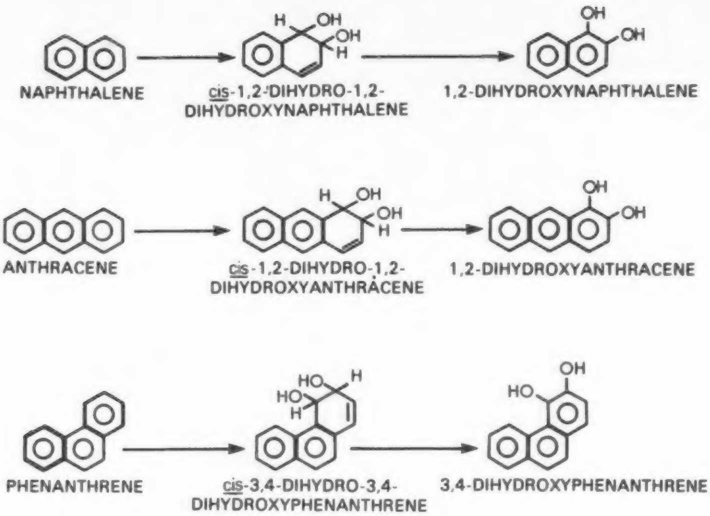

H
OH
OH
H
OH
OH
NAPHTHALENE
cis-1,2-DIHYDRO-1,2-DIHYDROXYNAPHTHALENE
1,2-DIHYDROXYNAPHTHALENE
H
OH
OH
H
OH
OH
ANTHRACENE
cis-1,2-DIHYDRO-1,2-DIHYDROXYANTHRACENE
1,2-DIHYDROXYANTHRACENE
HO
H
HO
H
HO
OH
PHENANTHRENE
cis-3,4-DIHYDRO-3,4-DIHYDROXYPHENANTHRENE
3,4-DIHYDROXYPHENANTHRENE

Figure 20. Mechanism of bacterial attack on naphthalene, anthracene, and phenanthrene.

Adapted from References 145, 224.

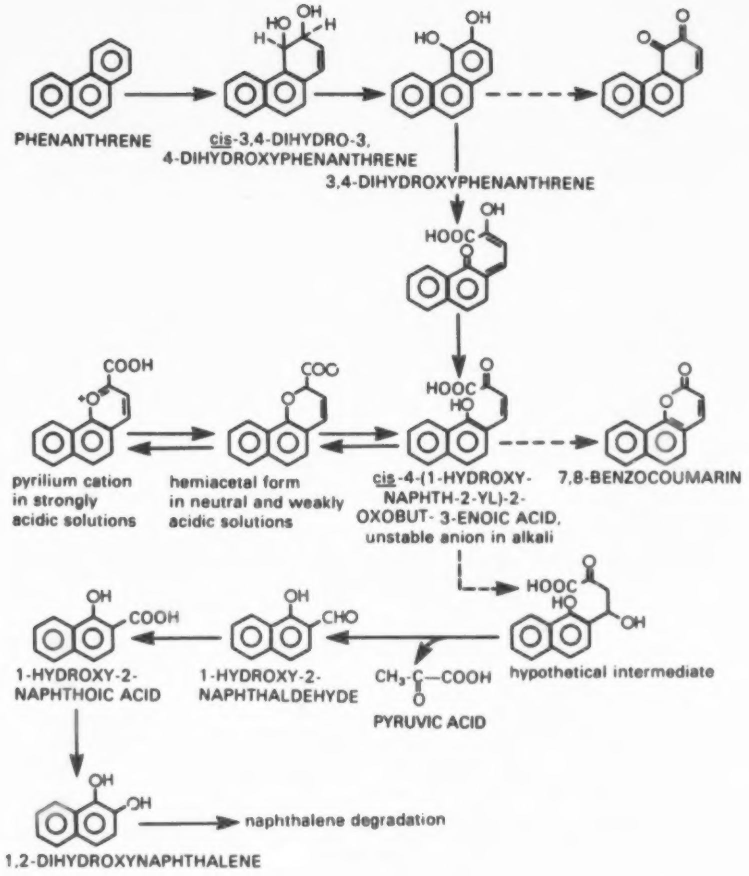

PHENANTHRENE
cis-3,4-DIHYDRO-3, 4-DIHYDROXYPHENANTHRENE
HO
OH
H
H
HO
OH
O
O
3,4-DIHYDROXYPHENANTHRENE
OH
HOOC
O
COOH
COO
HOOC
O
HO
O
pyrilium cation in strongly acidic solutions
hemiacetal form in neutral and weakly acidic solutions
cis-4-(1-HYDROXY-NAPHTH-2-YL)-2-OXOBUT- 3-ENOIC ACID, unstable anion in alkali
7,8-BENZOCOUMARIN
HOOC
HO
OH
hypothetical intermediate
OH
COOH
1-HYDROXY-2-NAPHTHOIC ACID
OH
CHO
1-HYDROXY-2-NAPHTHALDEHYDE
CH₃-C—COOH
O
PYRUVIC ACID
OH
OH
naphthalene degradation
1,2-DIHYDROXYNAPHTHALENE

Figure 21. Pathway of phenanthrene metabolism by *Pseudomonas* sp. Dashed lines indicate hypothetical pathways.
Adapted from Reference 145.

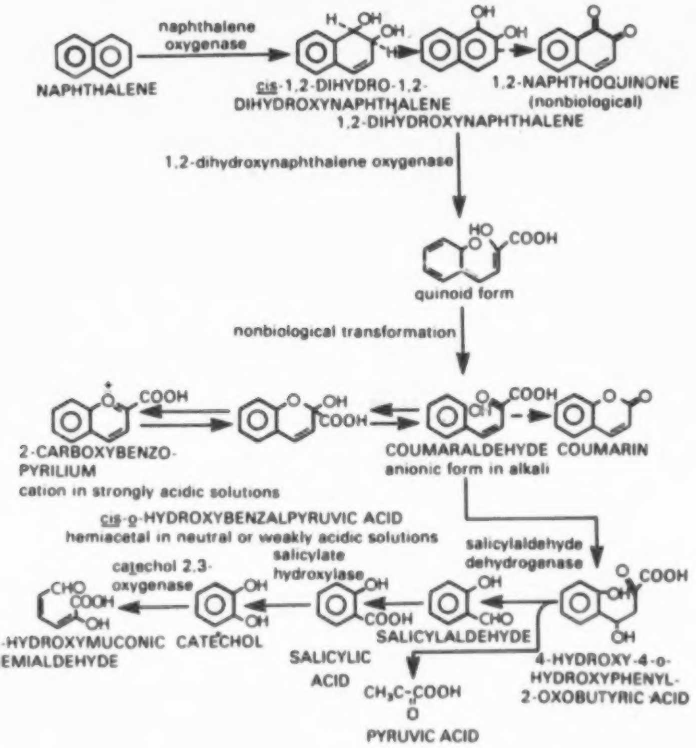

naphthalene oxygenase
NAPHTHALENE
cis-1,2-DIHYDRO-1,2-DIHYDROXYNAPHTHALENE
1,2-DIHYDROXYNAPHTHALENE
1,2-NAPHTHOQUINONE (nonbiological)
1,2-dihydroxynaphthalene oxygenase
HO
COOH
quinoid form
nonbiological transformation
COOH
2-CARBOXYBENZO-PYRILIUM
cation in strongly acidic solutions
OH
COOH
cis-o-HYDROXYBENZALPYRUVIC ACID
hemiacetal in neutral or weakly acidic solutions
COOH
COUMARALDEHYDE
anionic form in alkali
COUMARIN
salicylaldehyde dehydrogenase
COOH
OH
4-HYDROXY-4-o-HYDROXYPHENYL-2-OXOBUTYRIC ACID
OH
CHO
SALICYLALDEHYDE
salicylate hydroxylase
OH
COOH
SALICYLIC ACID
catechol 2,3-oxygenase
OH
OH
CATECHOL
CHO
COOH
OH
2-HYDROXYMUCONIC SEMIALDEHYDE
CH₃C-COOH
O
PYRUVIC ACID

Figure 22. Pathway of naphthalene metabolism by *Pseudomonas* spp. Dashed lines indicate hypothetical pathways.

Adapted from Reference 106.

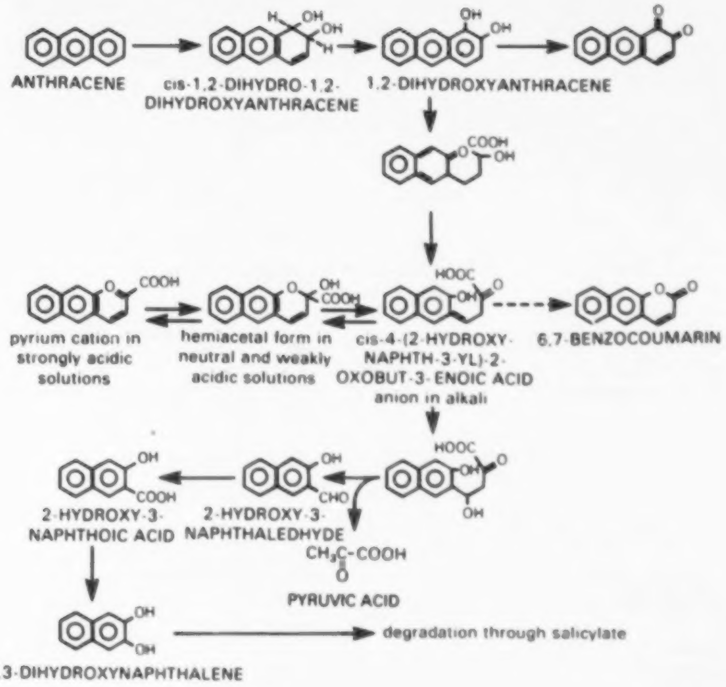

ANTHRACENE
cis-1,2-DIHYDRO-1,2-DIHYDROXYANTHRACENE
1,2-DIHYDROXYANTHRACENE
H
OH
OH
H
OH
OH
O
O
COOH
OH
COOH
O
pyrium cation in strongly acidic solutions
OH
COOH
O
hemiacetal form in neutral and weakly acidic solutions
HOOC
OH
O
cis-4-(2-HYDROXY-NAPHTH-3-YL)-2-OXOBUT-3-ENOIC ACID anion in alkali
O
O
6,7-BENZOCOUMARIN
HOOC
OH
O
OH
OH
CHO
2-HYDROXY-3-NAPHTHALEDHYDE
CH₃C-COOH
O
PYRUVIC ACID
OH
COOH
2-HYDROXY-3-NAPHTHOIC ACID
OH
OH
2,3-DIHYDROXYNAPHTHALENE
degradation through salicylate

Figure 23. Pathway of anthracene metabolism by *Pseudomonas* spp. Dashed lines indicate hypothetical pathways.

Adapted from Reference 145.

ATTACK ON AROMATIC STRUCTURES BY CYANOBACTERIA

The cyanobacteria have not been studied as extensively with regard to aromatic degradation. An investigation of the degradation of naphthalene by the cyanobacterium *Oscillatoria* sp. revealed the presence of 1-naphthol as the major metabolite (Figure 24); 57% of the 1-naphthol formed involved the incorporation of molecular oxygen (76a, 78a). Small amounts of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene were recovered which readily dehydrated to form 1-naphthol. *Trans*-1,2-dihydroxy-1,2-dihydronaphthalene was not recovered. There are three possible pathways which suggest a mechanism for attack of cyanobacteria on the aromatic ring: (a) similar to that for mammalian systems (path I, Figure 24), (b) unique to photosynthetic organisms (path II), or (c) similar to that for heterotrophic bacteria (path III) (78a).

RING FISSION OF DIHYDROXY AROMATIC COMPOUNDS BY BACTERIA

Bacteria can open an aromatic ring containing two hydroxyl groups if the groups are located *ortho* (adjacent) or *para* (opposite) to each other. If the groups are *ortho*, ring cleavage can occur either between the two (*ortho* cleavage) or next to one group (*meta* cleavage). The choice of which pathway is induced depends partly on the substrate and partly on the genetic constitution of the particular bacterial species. Both pathways can be induced independently of each other. An organism may utilize one pathway preferentially although it contains the enzymes for both pathways. For example, *P. putida (arvilla)* mt-2 metabolizes catechol via the *meta* pathway, although it contains the enzymes for both the *meta* and *ortho* pathways (323).

The *ortho* cleavage pathway of catechol leads to formation of 3-ketoadipic acid (Figure 25). Substituted catechols can be metabolized by an analogous path until either the substituent is expelled or the compound formed along the pathway cannot be metabolized further. Thus, the *ortho* pathway for protocatechuic acid dissimilation converges with that of catechol at 3-ketoadipate enol-lactone after the carboxyl group is expelled (Figure 26) (66, 84, 104). This latter compound is converted to 3-ketoadipic acid, which picks up coenzyme-A from succinyl-CoA to form the intermediate 3-ketoadipyl-CoA. Cleavage of this compound results in acetyl-CoA and succinyl-CoA which in turn exchanges its coenzyme-A with 3-ketoadipyl-CoA leaving one molecule of succinic acid (408). These aliphatic molecules enter the cell's tricarboxylic acid cycle.

The *meta* cleavage pathway (Figure 25) results in the formation of pyruvic acid and acetaldehyde from catechol (102, 103, 180, 330). Substituted catechols usually form pyruvic acid and another aldehyde or an acid (Figure 26). The substrate 3-methyl catechol yields acetate while 4-methyl catechol yields formic acid (27). The nonfluorescent pseudomonad group metabolizes protocatechuate by the *meta* pathway, utilizing protocatechuate 4,5-oxygenase with subsequent production of oxaloacetic acid and pyruvic acid. *Meta* cleavage of protocatechuic acid in *Bacillus* spp. is catalyzed by protocatechuate 2,3-oxygenase and yields pyruvic acid and acetaldehyde via the 2-hydroxymuconic semialdehyde intermediate (95). *Meta* cleavage degradation of catechol results in two possible routes of metabolism as demonstrated in *Azotobacter* sp. and *P. putida* NCIB 10015 (382, 383), the major route involving an NAD^+ dependent dehydrogenase and resulting in formation of 4-oxalocrotonic acid and the minor route not requiring NAD^+ but rather employing a hydrolase to form 2-oxopent-4-enoic acid directly. The paths converge at this step. Degradation of 3-methylcatechol is constrained to follow the hydrolase pathway only, as the NAD^+ dependent path requires the presence of an aldehyde group on the molecule. Other

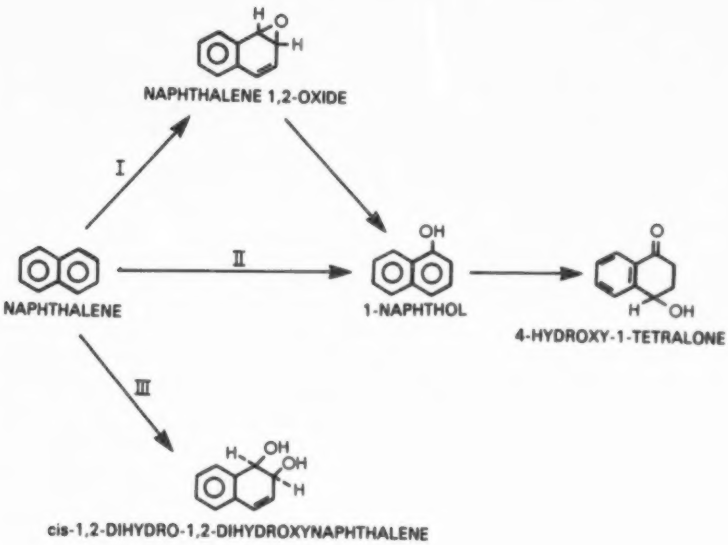

NAPHTHALENE 1,2-OXIDE
I
NAPHTHALENE
II
OH
1-NAPHTHOL
4-HYDROXY-1-TETRALONE
III
cis-1,2-DIHYDRO-1,2-DIHYDROXYNAPHTHALENE

Figure 24. Pathways for naphthalene metabolism by *Oscillatoria* sp., strain JCM. (I) Metabolism via 1,2-oxide; (II) light-dependent direct hydroxylation of naphthalene; (III) metabolism via dihydrodiol.

Adapted from Reference 78a.

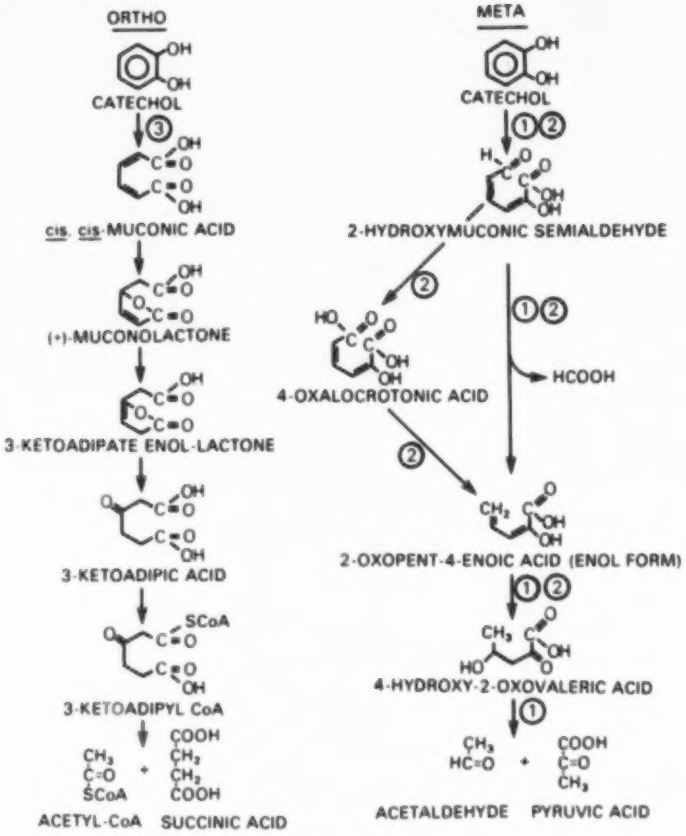

ORTHO
CATECHOL
③
cis, cis-MUCONIC ACID
(+)-MUCONOLACTONE
3-KETOADIPATE ENOL-LACTONE
3-KETOADIPIC ACID
SCoA
3-KETOADIPYL CoA
CH₃ C=O SCoA
COOH CH₂ CH₂ COOH
ACETYL-CoA
SUCCINIC ACID
META
CATECHOL
①②
2-HYDROXYMUCONIC SEMIALDEHYDE
②
4-OXALOCROTONIC ACID
①②
HCOOH
②
2-OXOPENT-4-ENOIC ACID (ENOL FORM)
①②
4-HYDROXY-2-OXOVALERIC ACID
①
CH₃ HC=O
COOH C=O CH₃
ACETALDEHYDE
PYRUVIC ACID

Figure 25. *Ortho-* and *meta-*cleavage pathways of catechol metabolism by bacteria. Adapted from References (1) 27, 103; (2) 382, 382a, 383, 468a; (3) 340, 408.

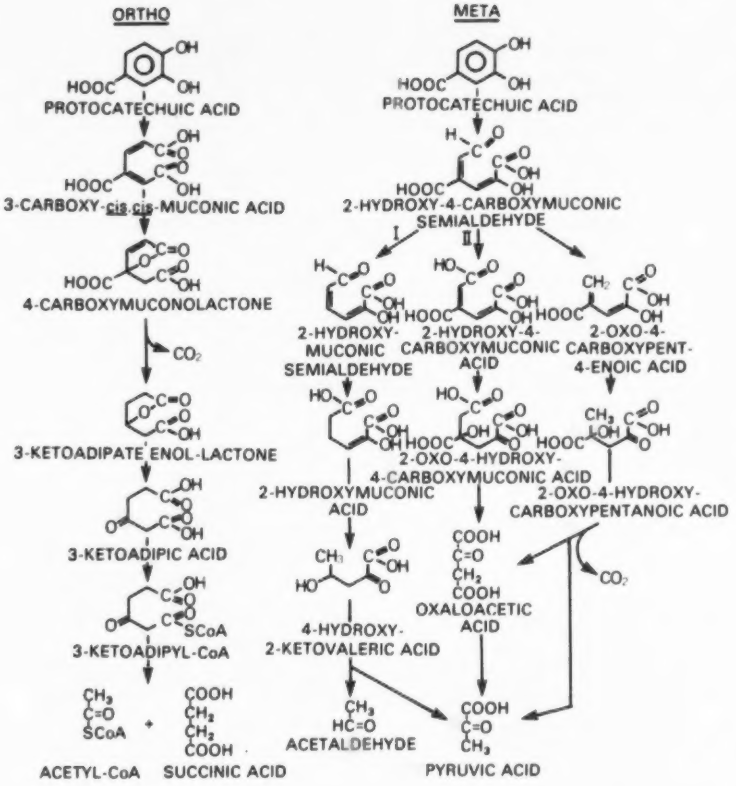

ORTHO
META
PROTOCATECHUIC ACID
3-CARBOXY-cis,cis-MUCONIC ACID
4-CARBOXYMUCONOLACTONE
CO2
3-KETOADIPATE ENOL-LACTONE
3-KETOADIPIC ACID
3-KETOADIPYL-CoA
ACETYL-CoA
SUCCINIC ACID
PROTOCATECHUIC ACID
2-HYDROXY-4-CARBOXYMUCONIC SEMIALDEHYDE
I
II
2-HYDROXY-MUCONIC SEMIALDEHYDE
2-HYDROXY-4-CARBOXYMUCONIC ACID
2-OXO-4-CARBOXYPENT-4-ENOIC ACID
2-HYDROXYMUCONIC ACID
2-OXO-4-HYDROXY-4-CARBOXYMUCONIC ACID
2-OXO-4-HYDROXY-CARBOXYPENTANOIC ACID
4-HYDROXY-2-KETOVALERIC ACID
OXALOACETIC ACID
CO2
ACETALDEHYDE
PYRUVIC ACID

Figure 28. *Ortho-* and *meta-*cleavage pathways of protocathechuate metabolism by bacteria. (1) *Bacillus* sp., (II) *P. testosteroni.*

Adapted from References 66a, 95, 340, 408.

compounds which undergo *meta* cleavage include naphthalene, 2,3-dihydroxyphenylpropionic acid, 2,3-dihydroxybenzoic acid, and homoprotocatechuic acid (84, 106).

Compounds that contain two hydroxyl groups located opposite to each other (*para* substitution), such as homogentisic acid and gentisic acid, are usually cleaved at the bond between one hydroxyl and the adjacent side chain, leading to fumaric acid and acetoacetic acid or fumaric acid and pyruvic acid, respectively (Figure 27) (84).

Metabolism of benzoic acid by *P. testosteroni* and *P. acidovorans* follow divergent pathways with a different hydroxylase being induced in each one (Figure 28), resulting in *meta* and *para* cleavage pathways, respectively. *P. testosteroni* produces two moles of pyruvic acid and one of formic acid, while *P. acidovorans* induces the gentisic acid pathway and produces one mole each of fumaric acid and pyruvic acid (467).

Biphenyl hydroxylated at the carbon-2 and carbon-3 positions may be cleaved in the *meta* position in two ways. An unidentified Gram-negative organism and a mutant strain of *Beijerinckia* sp. metabolize this compound by cleavage between the carbon-3 and carbon-4 positions to yield 2-hydroxy-3-phenylmuconic semialdehyde and subsequently phenylpyruvic acid (183, 287). However, *P. putida* cleaves 2,3-dihydroxybiphenyl between the carbon-1 and carbon-2 positions to form 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid and then benzoic acid (70, 71).

The dihydroxy fused ring compounds are cleaved initially by *meta* cleavage between the angular carbon and the adjacent hydroxyl. Hydroxynaphthalene is cleaved to form *cis*-o-hydroxybenzalpyruvic acid and subsequently salicylaldehyde, salicylic acid, and catechol (Figure 13). Catechol may be degraded by *ortho* or *meta* cleavage (106, 224, 255, 267, 353). In some bacteria, phenanthrene is dihydroxylated to *cis*-4-(1-hydroxy-naphth-2-yl)-2-oxobut-3-enoic acid, 1-hydroxy-2-naphthaldehyde, 1-hydroxy-2-naphthoic acid, and 1,2-dihydroxynaphthalene (Figure 12) (145, 151, 267, 373). Subsequent steps follow the pathway through salicylic acid. Other bacteria, including fluorescent and nonfluorescent pseudomonad groups, vibrios, and *Aeromonas* spp., metabolize phenanthrene to 1-hydroxy-2-naphthoic acid, 2-carboxybenzaldehyde, *o*-phthalic acid, and protocatechuic acid, which may undergo *ortho* or *meta* cleavage (255, 256). The first ring cleavage product of 1,2-dihydroxyanthracene is *cis*-4-(2-hydroxynaphth-3-yl)-2-oxobut-3-enoic acid which is degraded to 2-hydroxy-3-naphthaldehyde, 2-hydroxy-3-naphthoic acid and 2,3-dihydroxy-naphthalene (Figure 14) (145, 151, 267, 374). Degradation of 2,3-dihydroxynaphthalene continues through salicylic acid by an unknown pathway (145).

ATTACK OF AROMATIC STRUCTURES BY EUKARYOTES

The fate of aromatic-organic substances in mammalian systems has been studied extensively, both *in vivo* (injecting animals directly and recovering metabolites in body fluids or tissues) and using extracts of liver (or other) cells which contain the enzymes active in degradation of compounds (the microsomal enzymes). The mechanisms by which fungi and yeasts degrade aromatic compounds have been shown to be analogous to that of mammalian systems (404).

In contrast to bacteria, fungi utilize a monooxygenase which incorporates one atom of molecular oxygen into the benzene ring while converting the other to water. The resulting intermediate is an epoxide, which undergoes hydration with water to form a *trans*-1,2-dihydroxy-1,2-dihydro intermediate and subsequently a trans-dihydroxy compound (Figure 29) (175). Alternatively, the epoxide can isomerize to form phenols (177). Both of these mechanisms operate in the attack on naphthalene

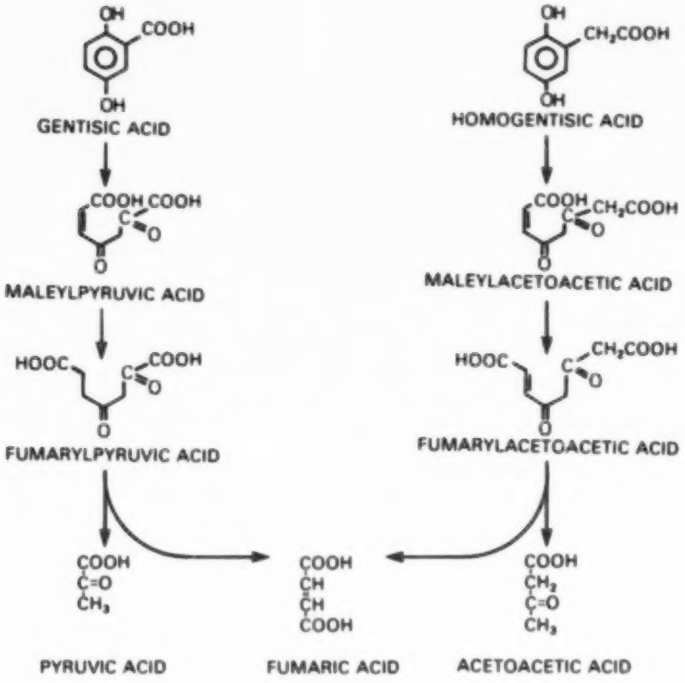

GENTISIC ACID
HOMOGENTISIC ACID
MALEYLPYRUVIC ACID
MALEYLACETOACETIC ACID
FUMARYLPYRUVIC ACID
FUMARYLACETOACETIC ACID
PYRUVIC ACID
FUMARIC ACID
ACETOACETIC ACID

Figure 27. Pathways of gentisic acid and homogentisic acid metabolism by bacteria. Adapted from References 84, 84a, 266a.

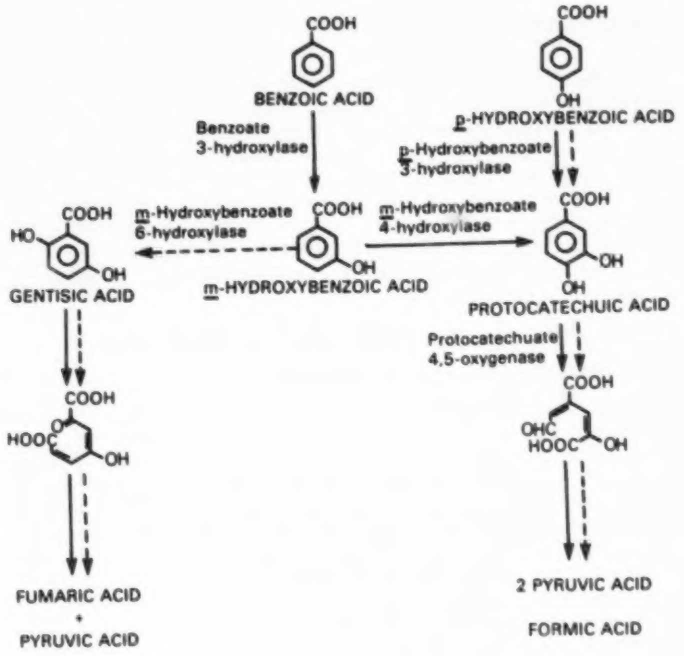

COOH
BENZOIC ACID
Benzoate
3-hydroxylase
COOH
OH
p-HYDROXYBENZOIC ACID
p-Hydroxybenzoate
3-hydroxylase
COOH
HO
OH
GENTISIC ACID
m-Hydroxybenzoate
6-hydroxylase
COOH
OH
m-HYDROXYBENZOIC ACID
m-Hydroxybenzoate
4-hydroxylase
COOH
OH
OH
PROTOCATECHUIC ACID
Protocatechuate
4,5-oxygenase
COOH
HOOC
OH
COOH
OHC
HOOC
OH
FUMARIC ACID
+
PYRUVIC ACID
2 PYRUVIC ACID
FORMIC ACID

Figure 28. Divergent pathways for the metabolism of benzoic acid, *p*-hydroxybenzoic acid, and *m*-hydroxybenzoic acid by *P. testosteroni*(solid arrows) and *P. acidovorans*(broken arrows). Adapted from Reference 467.

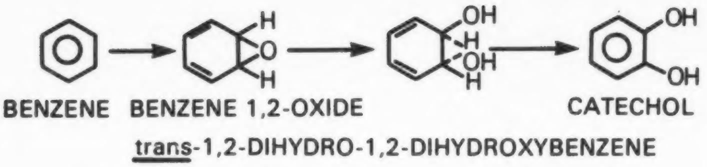


Figure 29. Formation of catechol from benzene in fungi, yeasts, and mammals.

by *Cunninghamella elegans* in which the primary metabolite is 1-naphthol (Figure 30) (76). Anthracene is oxidized by *C. elegans* predominantly to trans-1,2-dihydroxy-1,2-dihydroanthracene with formation of 1-anthryl sulfate (sulfate conjugation of 1-anthrol) as well (73). Other unidentified metabolites are also produced. Other compounds which have a hydroxyl group added during fungal metabolism include acetanilide, aniline, anisole, benzene, benzoic acid, biphenyl, and toluene (404). In some cases the position of the hydroxyl is variable or more than one hydroxyl group is added to the ring. For example, *C. elegans* hydroxylates biphenyl to 2-dihydroxybiphenyl, 3-hydroxybiphenyl, 4-hydroxybiphenyl, 2,4′-dihydroxybiphenyl, and 4,4′-dihydroxybiphenyl (116).

DEGRADATION OF DIHYDROXYLATED AROMATIC COMPOUNDS BY YEASTS AND FUNGI

In general, fungi and yeasts lack many of the ring fission dioxygenases characteristic of bacteria (5, 274). In most fungi and yeasts, catechol and hydroxyquinol are cleaved only by the *ortho* mechanism, utilizing 1,2-dioxygenases only (5, 327, 395). Phenol is metabolized through catechol by the *ortho* pathway (395). *Aspergillus niger* converts benzoic acid to benzaldehyde (359). A single strain of *Penicillium* sp. only has been found to utilize the *meta*-fission pathway (67). However, certain catabolic enzymes of the yeasts have broader substrate specificities than the equivalent bacterial enzymes. In addition, a third hydroxyl group can be introduced into the aromatic ring (5). Thus, although the yeast *Trichosporon cutaneum* lacks dioxygenases for protocatechuic acid, gentisic acid, and homoprotocatechuic acid, it can metabolize these substrates by means of NADH-dependent hydroxylases (Figure 31).

Methoxylated aromatic compounds are demethylated and converted to the corresponding hydroxybenzoic acids by microfungi (42) but are reduced to their corresponding aldehydes or alcohols by the wood-rotting basidiomycete *Polystictus versicolor* (147). The metabolism of protocatechuic acid through 3-carboxymuconic acid and 3-carboxymuconolactone to 3-ketoadipate by *Neurospora crassa* is typical of many fungi (67, 188). Protocatechuic acid is formed from p-hydroxybenzoic acid or *p*-methoxybenzoic acid. The protocatechuic acid 3,4-oxidase of *Rhodotorula mucilaginosa* was used to identify the first metabolite of protocatechuic acid as 3-carboxy-*cis*, *cis*-muconic acid (67). Fungi contain a lactonizing enzyme which converts this compound to 3-carboxymuconolactone. This is in contrast to bacteria, which form 4-carboxymuconolactone (93). The product of 3-carboxymuconolactone degradation is 3-ketoadipic acid.

Some groups of fungi form catechol from protocatechuic acid (67) via catechol 1,2-oxygenase. Further degradation leads to *cis,cis*-muconic acid and (+)-muconolactone via a *cis,cis*-muconic acid-lactonizing enzyme and a muconolactone. Eventually 3-ketoadipic acid is formed and subsequently metabolized to succinic acid and acetyl-CoA. The catechol pathway is similar to that of bacteria (67). Fungal

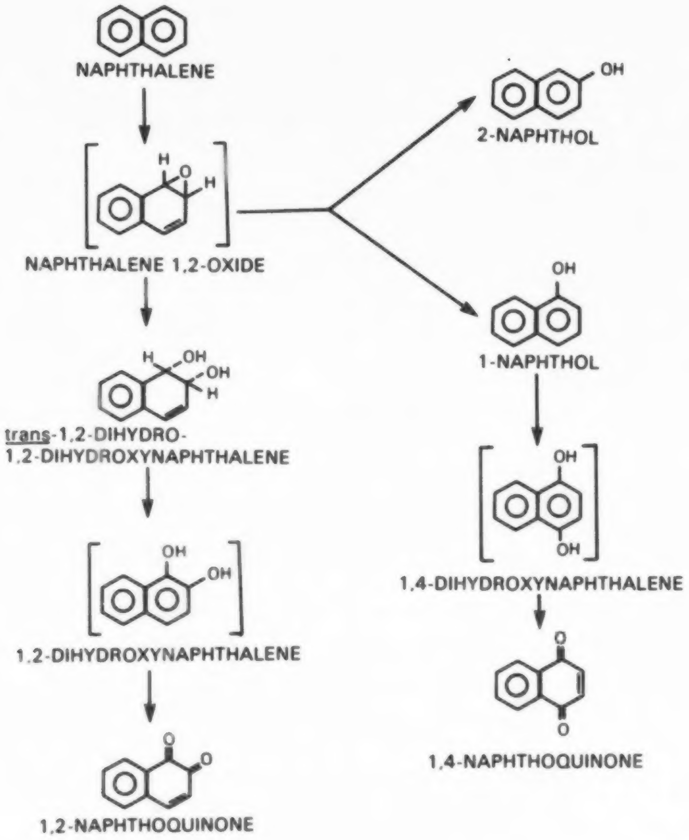

NAPHTHALENE
NAPHTHALENE 1,2-OXIDE
trans-1,2-DIHYDRO-
1,2-DIHYDROXYNAPHTHALENE
1,2-DIHYDROXYNAPHTHALENE
1,2-NAPHTHOQUINONE
2-NAPHTHOL
1-NAPHTHOL
1,4-DIHYDROXYNAPHTHALENE
1,4-NAPHTHOQUINONE
H
O
OH

Figure 30. Pathway of naphthalene metabolism by *C. elegans*. Adapted from Reference 76.

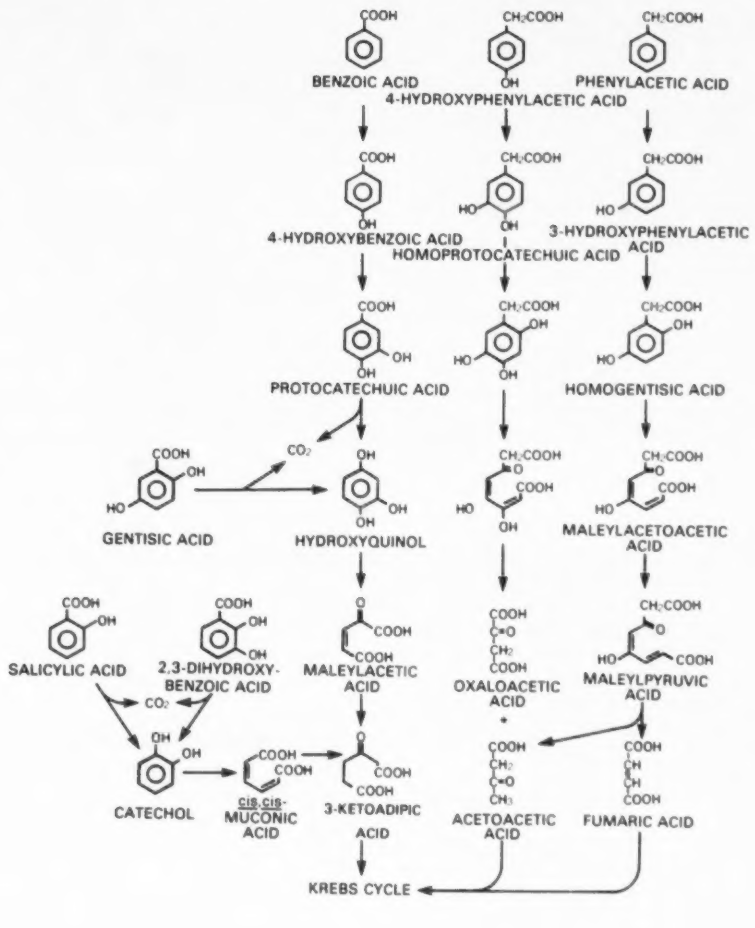

BENZOIC ACID
4-HYDROXYPHENYLACETIC ACID
PHENYLACETIC ACID
4-HYDROXYBENZOIC ACID
HOMOPROTOCATECHUIC ACID
3-HYDROXYPHENYLACETIC ACID
PROTOCATECHUIC ACID
HOMOGENTISIC ACID
CO2
GENTISIC ACID
HYDROXYQUINOL
MALEYLACETOACETIC ACID
SALICYLIC ACID
2,3-DIHYDROXY-BENZOIC ACID
MALEYLACETIC ACID
OXALOACETIC ACID
MALEYLPYRUVIC ACID
CO2
CATECHOL
CIS,CIS-MUCONIC ACID
3-KETOADIPIC ACID
ACETOACETIC ACID
FUMARIC ACID
KREBS CYCLE

Figure 31. Metabolism of aromatic compounds by *T. cutaneum*. Adapted from Reference 5.

metabolism of 1,2-dihydroxynaphthalene or 1-naphthol leads to 1,2-naphthoquinone or 1,4-naphthoquinone, respectively (75, 76, 78). These pathways are similar to those of mammalian microsomal extracts and are due to the cytochrome P-450 present in some fungi as well as in mammals (75). Monohydroxylated biphenyl compounds are hydroxylated further to various dihydroxy compounds (184a). These transformations are similar to those of mammals (184a, 404).

SUMMARY

Bacteria and eukaryotes differ fundamentally in the mechanism of primary oxidation of aromatic compounds. Bacteria usually add two atoms of molecular oxygen from the same atmospheric oxygen molecule using a dioxygenase enzyme. The mechanism of the oxidative addition results in a *cis*-1,2-dihydrodiol intermediate. In a few cases, such as when the aromatic ring is already monooxygenated (as for phenol), a hydroxylase (a monooxygenase enzyme) is utilized. The *ortho*-dihydrodiol molecules are subject to ring cleavage by either of two mechanisms. *Ortho* cleavage enzymes such as catechol 1,2-oxygenase open the ring between the adjacent hydroxyl groups. The molecule subsequently is metabolized via *cis*, *cis*-muconic acid and 3-ketoadipic acid to acetyl-CoA and succinic acid. In contrast, the *meta* cleavage pathway opens the ring adjacent to one hydroxyl group using enzymes such as catechol 2,3-oxygenase. The intermediate compounds of this pathway include 3-hydroxymuconic acid, and the end products include pyruvic acid and an aldehyde. Compounds such as benzoic acid initially may be monohydroxylated in the *meta* position. A second hydroxylase may then form a *para*-dihydroxylated molecule which is metabolized via the gentisic acid pathway to fumaric acid and pyruvic acid. An alternative pathway of benzoic acid dissimilation attributable to bacteria is demonstrated in anaerobic reductive metabolism by *R. palustris* which forms pimelic acid via the cyclohexanol intermediate.

Fused-ring compounds are *ortho*-dihydroxylated at the positions adjacent to the angular carbon, and are cleaved by the *meta* pathway.

Cyanobacteria appear to attack aromatic structures by a mechanism similar to that of heterotrophic bacteria, which results in a *cis*-dihydrodiol intermediate. However, the exact mechanism has not been elucidated. Fungi and other eukaryotes attack benzene structures by monooxygenases which incorporate one atom of molecular oxygen, forming an epoxide. Subsequently the epoxide may undergo hydration with water to form a *trans*-1,2-dihydrodiol intermediate. Alternatively, a monohydroxylated compound may be formed.

The *ortho* mechanism is most commonly utilized by fungi for ring fission. The protocatechuic acid pathway in fungi differs from that of bacteria in that 3-carboxy-*cis,cis*-muconic acid is lactonized to the 3-lactone in fungi and the 4-lactone in bacteria. Fungi lack many of the substrate-specific dioxygenases characteristic of bacteria, but some of their catabolic enzymes have broader substrate specificities than the equivalent bacterial enzymes. A third hydroxyl group may be added to the ring by fungi to facilitate metabolism of a molecule. The similarity in metabolism by fungi and mammals of many aromatic compounds has been demonstrated by comparison of the resultant metabolites (Table 3) (404). Thus, fungi may serve as models for many mammalian metabolic mechanisms.

TABLE 3. COMPARISON OF METABOLITES FORMED BY EUKARYOTES AND MAMMALIAN SYSTEMS*

		Mammalian metabolites	
Substrate	Fungal metabolites	In vitro	In vivo
Aniline	Acetanilide, 2-hydroxyaetanilide, and 4-hydroxyaniline	4-Hydroxyaniline	Acetanilide, 2-, 3-, and 4-hydroxyaniline
Anisole	2- and 4-Hydroxy anisole, phenol	2- and 4-Hydroxy anisole, phenol	2- and 4-Hydroxy anisole
Benzene	Phenol	Phenol	Phenol
Benzoic acid	2- and 4-Hydroxybenzoic acid, 3,4-dihydroxybenzoic acid	3-Hydroxybenzoic acid	2-, 3-, and 4-hydroxybenzoic acid
Biphenyl	2- and 4-Hydroxybiphenyl, 4,4'-dihydroxybiphenyl	2- and 4-Hydroxybiphenyl	4-Hydroxy-, 3,4-dihydroxy- and 4,4'-dihydroxy biphenyl
Chlorobenzene	2- and 4-Hydroxychlorobenzene	2-, 3-, and 4-Hydroxychlorobenzene	2-, 3-, and 4-Hydroxybenzene
Naphthalene	1- and 2-Hydroxynaphthalene	1- and 2-Hydroxynaphthalene	1- and 2-Hydroxynaphthalene
Toluene	2- and 4-Hydroxy toluene	2- and 4-Hydroxy toluene, benzyl alcohol	Benzoic acid and conjugates

*Adapted from reference 404.

SECTION 7

CHLOROBENZOIC ACIDS

Chlorobenzoic acids are introduced into the environment as degradative products of polychlorinated biphenyls (1, 334a) and herbicides as well as in direct application as herbicides (41). For example, the multisubstituted herbicide 2,3,6-trichlorobenzoic acid is a growth regulator similar in function to 2,4-dichlorophenoxyacetic acid (208).

BACTERIAL METABOLISM AND CHLOROBENZOIC ACIDS

The degradation of 2,3,6-trichlorobenzoic acid (Figure 32) has been investigated using resting cell suspensions of *Brevibacterium* sp. grown on benzoic acid (208, 210). The major resulting product is 3,5-dichlorocatechol which appears with stoichiometric release of one mole of chloride and one mole of CO_2 per mole of herbicide metabolized (208). The initial oxidation of 2,3,6-trichlorobenzoic acid takes place at the unsubstituted carbon-4 position. This is followed by a one-step oxidation-dechlorination at the adjacent chlorinated carbon. The pathway thus proceeds through 2,3,6-trichloro-4-hydroxybenzoic acid to 2,3,5-trichlorophenol and subsequently to 3,5-dichlorocatechol (Figure 32). The dichlorocatechol accumulates in the medium and is toxic to the *Brevibacterium* sp. cells. However, resting cell suspensions of *Achromobacter* sp. grown on benzoic acid will cleave 3,5-dichlorocatechol by the *meta* pathway to form 2-hydroxy-3,5-dichloromuconic semialdehyde (210). This metabolite accumulates and is toxic to the *Achromobacter* sp. cells.

Several species of bacteria have been shown to metabolize 3-chloro- and 4-chlorobenzoic acid. Resting cell suspensions of *Arthrobacter* sp. grown on benzoic acid oxidize 3-chlorobenzoic acid to 4-chlorocatechol, which is not inhibitory to growth or oxygen uptake (210, 211). *Pseudomonas aeruginosa* strain B23 accumulates 3-chlorocatechol from the metabolism of 3-chlorobenzoic acid (216). *Acinetobacter calcoaceticus* strain Bs5 grown on succinic acid or pyruvic acid will cometabolize 3-chlorobenzoic acid to both 3-chloro- and 4-chlorocatechol, which accumulate and are toxic (362). When mixtures of chlorocatechols can be formed, 3-chlorocatechol is the major metabolite (261, 362). *Meta* cleavage of 3-chlorocatechol results in an acylhalide which acts as an acylating agent and inactivates the *meta* pyrocatechase (catechol-cleaving enzyme) irreversibly, resulting in the lethal accumulation of catechols (261). Inefficient *ortho* cleavage will also result in the accumulation of chlorocatechols. *Meta* cleavage of 4-chlorocatechol yields 5-chloro-2-hydroxymuconic semialdehyde. Corresponding chlorocatechols are also formed from 3-chlorobenzoic acid and 4-chlorobenzoic acid by *Azotobacter* sp. grown on benzoic acid (414) and by *Pseudomonas* sp. WR912 (195).

Cells grown on chlorinated compounds including 3-chlorobenzoic acid are induced to produce high levels of pyrocatechase II, which has high activity against chlorocatechols as compared to catechols (118). Cells grown on nonchlorinated substrates express only pyrocatechase I, which does not function in chlorocatechol oxygenation. Pyrocatechases I and II are separate catechol 1,2-dioxygenases (118). Pyrocatechase I is involved in the degradation of catechol via the 3-ketoadipic acid pathway (117). Pyrocatechase II is similar to the *Brevibacterium* spp. pyrocatechase

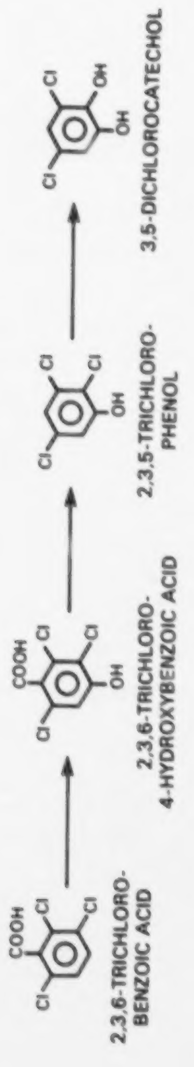

COOH
Cl
Cl
Cl
2,3,6-TRICHLORO-
BENZOIC ACID
COOH
Cl
Cl
Cl
OH
2,3,6-TRICHLORO-
4-HYDROXYBENZOIC ACID
Cl
Cl
Cl
OH
2,3,5-TRICHLORO-
PHENOL
Cl
Cl
OH
OH
3,5-DICHLOROCATECHOL

Figure 32. Formation of 3,5-dichlorocatechol from 2,3,6-trichlorobenzoic acid by *Brevibacterium* sp.

Adapted from Reference 208.

(321) and is unusual in its broad substrate specificity (118). The ability to cleave chlorocatechols, which are toxic, appears to be a crucial factor in the ability of microorganisms to degrade chloroaromatic compounds (118).

In cells of *Pseudomonas* sp. WR912, pyrocatechases I and II are both induced when the growth substrate is unsubstituted benzoic acid (195). This organism can use benzoic acid, 3-chloro-, 4-chloro-, and 3,5-dichlorobenzoic acids as sole sources of carbon and energy. Each is metabolized to the corresponding chlorocatechol, which undergoes *ortho* cleavage to form the chlorinated muconic acid. The muconic acid in each case is cycloisomerized with coincident or subsequent stoichiometric elimination of the chloride ion (Figure 33). Because of the wide range of substrates, the benzoic acid 1,2-dioxygenase of *Pseudomonas* sp. WR912 is characterized as being of low substrate specificity and also not stereospecific, similar to the corresponding enzyme of *P. putida* mt-2 (195).

In contrast, the benzoic acid 1,2-dioxygenase of *Pseudomonas* sp. B13 (Figure 34) shows narrow substrate specificity, as this organism metabolizes only 3-chlorobenzoic acid (195). The isomer 2-chlorobenzoic acid does not induce oxygen uptake, and 4-chlorobenzoic acid is oxidized only at very low reaction rates (363). Cells grown on 3-chlorobenzoic acid are adapted simultaneously to metabolize benzoic acid, but the reverse is not true (119). The enzymes of the 3-keto-adipic acid pathway are induced in cells grown on benzoic acid, and the chlorinated catechols accumulate. In contrast, resting cell suspensions of cultures grown on 3-chlorobenzoic acid produce both 3-chloro- and 5-chlorodihydroxybenzoic acid in almost equal quantities. A branched pathway thus exists for metabolism of 3-chlorobenzoic acid by *Pseudomonas* sp. B13 (Figure 34). Along one branch 3-chlorodihydroxybenzoic acid is metabolized to 3-chlorocatechol and along a parallel branch 5-chlorodihydroxybenzoic acid is converted to 4-chlorocatechol. The common enzyme involved, 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid dehydrogenase, has the same relative activity in both benzoic acid-grown and 3-chlorobenzoic acid-grown cells (365). The chlorocatechols are metabolized to muconic acids by pyrocatechases which are induced only in 3-chlorobenzoic acid-grown cells. The muconate cycloisomerase II enzyme which acts on the muconic acids to perform cycloisomerization has much higher activity in 3-chlorobenzoic acid-grown cells (119). Combined dechlorination and regeneration of the diene system is a spontaneous secondary reaction (386). *P. putida* strain 87 isolated from soils treated with pesticides also contains two pyrocatechases, one specific for the nonchlorinated catechol and the other specific for chlorinated catechols (187). The former is controlled by chromosomal genes and the latter is plasmid mediated. Chloromuconic acid was detected upon incubation of this strain with 3-chlorobenzoic acid.

The mutant strain *Alcaligenes eutrophus* B9 also produces 3-chloro- and 5-chlorodihydroxybenzoic acid from cooxidation of 3-chlorobenzoic acid (367). A strain of *P. putida*, harboring a plasmid termed pAC25, degrades 3-chlorobenzoic acid via 3-chlorocatechol and 3-chloromuconic acid (86). Chloride is released and maleylacetic acid rather than 3-ketoadipic acid is produced. This pathway is analogous to one path of 3-chlorobenzoic acid metabolism by *Pseudomonas* sp. B13.

Four strains of *Pseudomonas* spp. which utilize 3-chlorobenzoic acid as the sole source of carbon and energy for growth were isolated from sewage which had been enriched with the substrate (194). One isolate studied in detail, *Pseudomonas* sp. strain H1, resembles *Pseudomonas* sp. B13 in metabolism of 3-chlorobenzoic acid and benzoic acid, and therefore seems to possess both pyrocatechases. A black pigment, resulting from oxidation and polymerization of unmetabolized catechols, forms when *Pseudomonas* sp. strain H1 is incubated with both 3-chlorobenzoic acid and benzoic acid without prior adaptation to 3-chlorobenzoic acid. In contrast, another species isolated from the sewage enrichment culture metabolizes catechols and chlorocatechols rapidly enough to prevent occurrence of the black pigment.

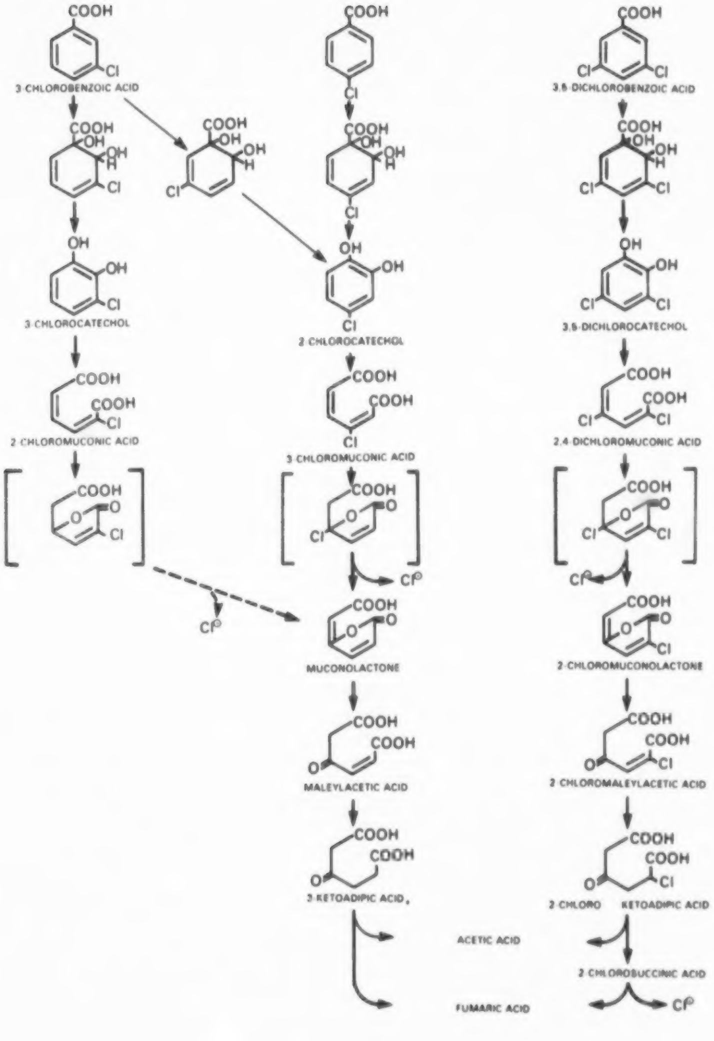

3-CHLOROBENZOIC ACID
3,5-DICHLOROBENZOIC ACID
3-CHLOROCATECHOL
2-CHLOROCATECHOL
3,5-DICHLOROCATECHOL
2-CHLOROMUCONIC ACID
3-CHLOROMUCONIC ACID
2,4-DICHLOROMUCONIC ACID
Cl⁻
Cl⁻
Cl⁻
MUCONOLACTONE
2-CHLOROMUCONOLACTONE
MALEYLACETIC ACID
2-CHLOROMALEYLACETIC ACID
3-KETOADIPIC ACID,
2-CHLORO KETOADIPIC ACID
ACETIC ACID
2-CHLOROSUCCINIC ACID
FUMARIC ACID
Cl⁻

Figure 33. Pathways of metabolism of chlorobenzoic acids by *Pseudomonas* sp. WR912. Compounds in brackets are hypothetical intermediates. Adapted from Reference 195.

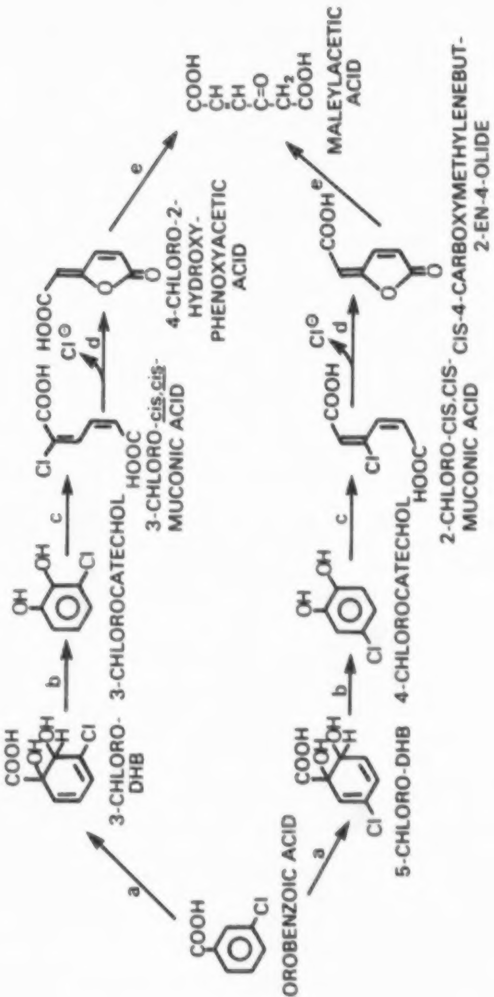

3-CHLOROBENZOIC ACID
3-CHLORO-DHB
3-CHLOROCATECHOL
3-CHLORO-cis,cis-MUCONIC ACID
4-CHLORO-2-HYDROXY-PHENOXYACETIC ACID
5-CHLORO-DHB
4-CHLOROCATECHOL
2-CHLORO-CIS,CIS-MUCONIC ACID
CIS-4-CARBOXYMETHYLENEBUT-2-EN-4-OLIDE
MALEYLACETIC ACID
a
b
c
d
e
COOH
Cl
OH
H
HOOC
Cl⊖
O
CH
C=O
CH₂

Figure 34. Metabolism of 3-chlorobenzoic acid by *Pseudomonas* sp. B13. DHB = 2,5, cyclohexadiene-1,2-diol-1-carboxylic acid. Enzymes: (a) benzoate dioxygenase; (b) DHB dehydrogenase; (c) pyrocatechase II; (d) muconate isomerase II; (e) 4-carboxymethylenebut-2-en-4-olide hydrolase.

Adapted from References 119, 386, 386a.

A hybrid strain has been developed which combines the *ortho* cleavage pathway of *Pseudomonas* sp. B13 with the relatively nonspecific toluate 1,2-dioxygenase of *P. putida* mt-2 (364). This derivative of *Pseudomonas* sp. B13 acquired the TOL plasmid from *P. putida* mt-2. In the resulting cells, both 4-chlorobenzoic acid and 3,5-dichlorobenzoic acid as well as 3-chlorobenzoic acid are metabolized (Figure 35). The enzyme, toluate 1,2-dioxygenase from the genes on the plasmid, is induced and slightly modified to result in increased turnover of the chlorinated compound used as the selective substrate. Dihydrodihydroxybenzoic acid dehydrogenases from both plasmid and chromosomal sources are induced. In addition, *ortho* pyrocatechases I and II are induced, but not the unproductive *meta* pyrocatechase.

A *Bacillus* sp. grown on benzoic acid uses a unique pathway to cometabolize 3-chlorobenzoic acid to 5-chloro-2,3-dihydroxybenzoic acid via 5-chlorosalicylic acid (406). Another unique pathway involving enzymatic rather than spontaneous elimination of chloride ion was demonstrated in *Bacillus brevis* isolated from polluted river water (96). This organism utilizes 5-chloro-2-hydroxybenzoic acid (5-chlorosalicylic acid) as a sole carbon and energy source. The first step in metabolism is cleavage between the carbon-1 and carbon-2 by a specific 5-chlorosalicylate 1,2-dioxygenase. This enzyme requires a halogen (except iodine) or a methoxyl group (but not a hydroxyl) at the carbon-5 position. Only one hydroxyl group is present on the molecule. After loss of the chloride ion and formation of maleylpyruvic acid, metabolism continues along the steps of the gentisic acid pathway (Figure 36).

A novel pathway for 3-chlorobenzoic acid metabolism in which the chloride is replaced by a hydroxyl group in the first step has been demonstrated in a *Pseudomonas* sp. isolated from soil (231). This organism utilizes 3-chlorobenzoic acid as a sole source of carbon for growth and metabolizes it to 3-hydroxybenzoic acid and subsequently to 2,5-dihydroxybenzoic acid.

Similarly, an *Arthrobacter* sp. growing on 4-chlorobenzoic acid as the sole source of carbon produces 4-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid (protocatechuic acid) (380). A strain of *Arthrobacter globiformis* also utilizes 4-chlorobenzoic acid as the sole carbon source and metabolizes it via 4-hydroxybenzoic acid and protocatechuic acid, with release of chloride (486). Direct replacement of the chloride by the hydroxyl group precludes formation of the potentially toxic chlorocatechol. *Pseudomonas* sp. strain CBS 3 also utilizes 4-chlorobenzoic acid as the sole source of carbon for growth (257) by the same pathway. The enzymes induced by growth with this substrate have been identified as 4-chlorobenzoate-4-hydroxylase, 4-hydroxybenzoate-3-hydroxylase, and protocatechuate-3,4-dioxygenase. The first enzyme was not induced by growth with 4-hydroxybenzoic acid or any of several other chlorinated and nonchlorinated substrates. The mechanism of action of this enzyme has not been elucidated. Protocatechuic acid was metabolized by the 3-ketoadipic acid pathway following *ortho* cleavage.

ALGAL METABOLISM OF CHLOROBENZOIC ACIDS

The only reference to algal metabolism of chlorobenzoic acids obtained involves a monoalgal culture of *Chlamydomonas* sp. strain A2 isolated from sewage (22[illegible]). The nonaxenic culture (bacteria present) transforms 4-chloro-3,5-dinitrobenzoic acid to 3-hydroxymuconic semialdehyde, indicating a *meta*-cleavage pathway. Approximately 20% chloride release was reported. Since the culture contained bacteria along with the algae, dechlorination might have been due to the action of the algae or it might have been a bacterial process with the algae providing fixed carbon or growth factors.

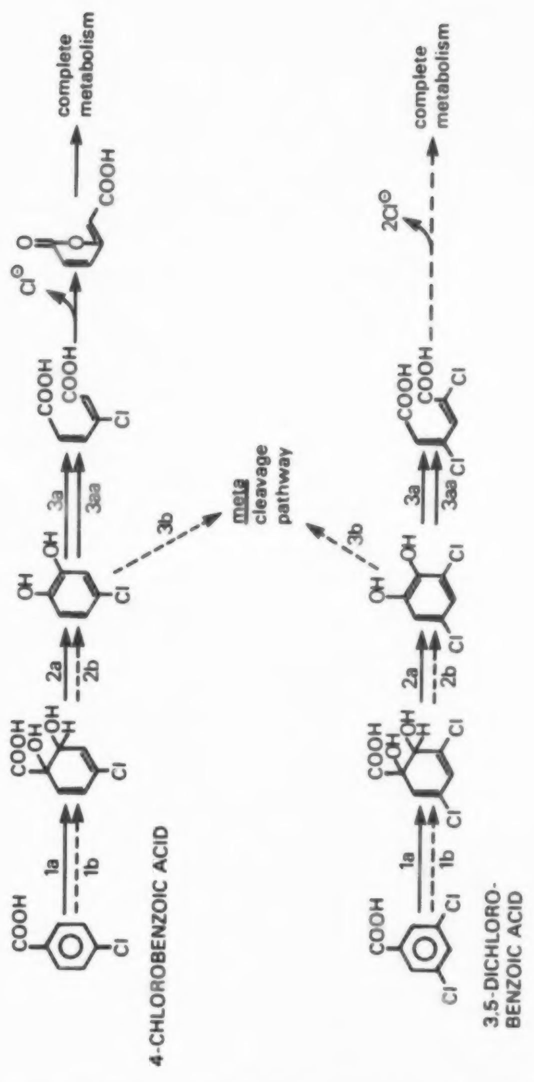

4-CHLOROBENZOIC ACID
COOH
Cl
1a
1b
OH
2a
2b
3a
3aa
3b
meta cleavage pathway
Cl⊖
O
complete metabolism
3,5-DICHLOROBENZOIC ACID
2Cl⊖

Figure 35. Metabolism of 4-chlorobenzoic acid and 3,5-dichlorobenzoic acid by *Pseudomonas* sp. B13 transconjugants. Enzymes marked (a) from *Pseudomonas* sp. B13; those marked (b) from donor *P. putida* mt-2. Enzymes: (1a) benzoate 1,2-dioxygenase; (1b) toluate 1,2-dioxygenase; (2a, 2b) dihydrodihydroxybenzoate dehydrogenase; (3a) pyrocatechase 1; (3aa) pyrocatechase II; (3b) *meta*-pyrocatechase. Adapted from Reference 464.

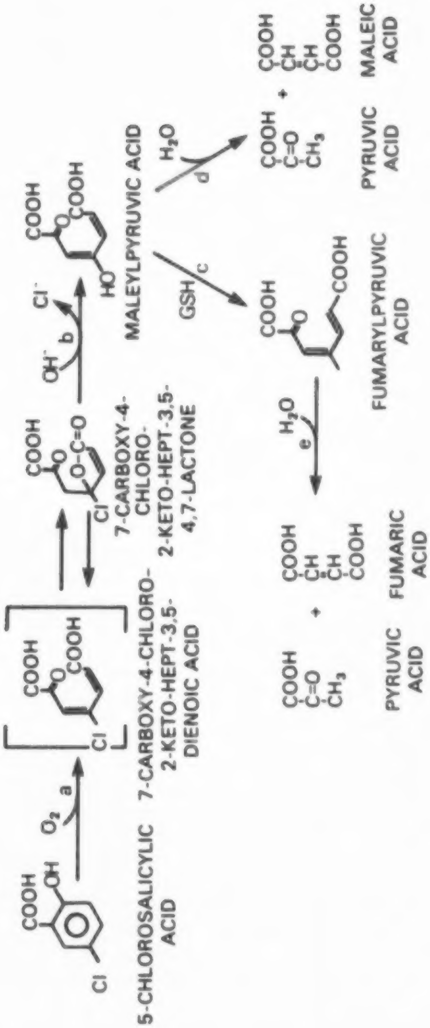

5-CHLOROSALICYLIC ACID
O2
a
7-CARBOXY-4-CHLORO-2-KETO-HEPT-3,5-DIENOIC ACID
7-CARBOXY-4-CHLORO-2-KETO-HEPT-3,5-4,7-LACTONE
OH⁻
b
Cl⁻
MALEYLPYRUVIC ACID
GSH
c
H2O
d
FUMARYLPYRUVIC ACID
H2O
e
PYRUVIC ACID
FUMARIC ACID
PYRUVIC ACID
MALEIC ACID

Figure 36. Metabolism of 5-chlorosalicylic acid by *B. brevis*. Enzymes: (a) 5-chlorosalicylic acid 1,2-dioxygenase; (b) "lactone hydrolase"; (c) maleylpyruvate isomerase; (d) meleylpyruvate hydrolase; (e) fumarylpyruvate hydrolase. GSH = reduced glutahione.

Adapted from Reference 96.

FUNGAL METABOLISM OF CHLOROBENZOIC ACIDS

Aspergillus niger cultures utilized both 2-chloro- and 3-chlorobenzoic acid as sole sources of carbon and energy (390). Protocatechuic acid and 4-hydroxybenzoic acid were isolated from both samples. Cells grown on these substrates oxidize all four compounds as well as benzoic acid more rapidly than do cells grown on glucose. Adapted cultures dechlorinate either substrate, while glucose grown cells do not have this capability. Dehalogenating activity was also noted in the cell-free extracts of cultures grown on 2-chlorobenzoic acid.

METABOLISM OF CHLOROBENZOIC ACIDS IN SOILS AND BY CONSORTIA

Under cometabolic conditions with glucose as the additional carbon source, a sewage plant effluent inoculum metabolized 3-chlorobenzoic acid with production of 3-chlorocatechol (212). Upon continued incubation this metabolite disappeared with concomitant appearance of 2-hydroxy-3-chloromuconic semialdehyde. After a 29-day period of no discernible metabolism, the semialdehyde was metabolized with appearance of stoichiometric amounts of inorganic chloride. There was no additional increase in cell numbers due to the presence of chlorobenzoic acid until degradation of the semialdehyde occurred.

Diluted wastewater sludge supernatant fluid mediated disappearance of 16 mg/l 3-chlorobenzoic acid within 14 days, although no degradation of 2- or 4-chlorobenzoic acid was seen after 25 days (193). Readaption of the sludge inocula to 3-chlorobenzoic acid greatly reduced the time required for metabolism of both 3- and 4-chlorobenzoic acid. Soil suspension also did not degrade 4-chlorobenzoic acid in 25 days, although the other 2 substrates were metabolized within 14 days. Application of 2,3,6-trichlorobenzoic acid to soil resulted in 30% chloride release within one month (113). No intermediate metabolites were detected.

A sewage microcosm enriched with chlorinated benzoic acids resulted in development of a consortium of Gram-negative motile rods and Gram-positive pleomorphic rods which could utilize as sole carbon and energy sources benzoic acid, 2-chloro-, 3-chloro-, 4-chloro- and 3,4-dichlorobenzoic acids but not 2,4-dichloro- or 2,3,6-trichlorobenzoic acid (114). Addition of biodegradable benzoic acids did not lead to decomposition of any of the substrates. Degradation of 3-chlorobenzoic acid led to formation of both 4-chlorocatechol and 5-chlorosalicylic acid, the latter compounds disappearing from solution. Formation of both metabolites indicates two separate pathways of metabolism within the consortium.

Pronamide [3,5-dichloro-N-(1,1-dimethyl-2-propynyl)benzamide] is an herbicide used for weed control on crops of lettuce and alfalfa and other legumes (153). Pronamide was metabolized in soils to $^{14}CO_2$ from both ^{14}C(carbonyl)- and ^{14}C(ring)-labeled substrate. In addition, seven other metabolites were found, none of which was dechlorinated. The potential metabolite 3,5-dichlorobenzoic acid was also metabolized with 80% of ^{14}C(carbonyl)- and 50% of ^{14}C(ring)-labeled substrate recovered as $^{14}CO_2$ within 28 days.

Dicamba (3,6-dichloro-2-methoxybenzoic acid) is used on wheat, oats, barley and flax crops for weed control. Studies with a temperature-gradient table showed that an increase in temperature from 5 to 35°C resulted in a linear increase in the disappearance rate for the herbicide in silty clay and heavy clay, while the range for a linear relationship of temperature to disappearance was 9 to 20°C for sandy loam, a soil with a lower moisture holding capacity (401). Studies with Regina heavy clay have shown that only 10% of the herbicide remains five weeks after application (399). The only radioactive metabolite recovered from application of carboxyl-group labeled dicamba was 3,6-dichlorosalicylic acid. About 20% of the substrate was recovered as $^{14}CO_2$, and metabolites which were not radioactive were not identified. Dicamba applied at a rate of 1.1 kg/ha to a silty clay with high organic content almost entirely disappeared within two weeks (399a). At the same application rate onto sandy loam and heavy clay, there was less than 10% substrate remaining after 4 weeks when the moisture content was high, although residues were still detected after 6 weeks in low-moisture soils. After 4 weeks, over 90% of the substrate applied to sterile soils was recovered. Studies with ^{14}C(carboxyl)- and ^{14}C(ring)-labeled dicamba revealed that 18% was released as $^{14}CO_2$ in 6 weeks and 45% in 17 weeks from ^{14}C(carboxyl)-dicamba, and 9% in 6 weeks from ^{14}C(ring)-dicamba (398). The only metabolite which could be recovered was 3,6-dichlorosalicylic acid.

REDUCTIVE DECHLORINATION

The chlorobenzoic acids have served as model substrates for the elucidation of anaerobic reductive dechlorination by consortia of bacteria from anaerobic sediment or sludge environments. This pathway for dechlorination of aromatic compounds involves removal of the aryl halide from the aromatic ring (Figure 37) (417). A consortium resulting from enrichment with 3-chlorobenzoic acid mineralizes this substrate through benzoic acid to methane and CO_2. The substrate 4-amino-3,5-dichlorobenzoic acid is converted to 4-amino-3-chlorobenzoic acid by replacement of one chlorine atom with a hydrogen atom. No chloride shift takes place. Neither 2-chloro- nor 4-chlorobenzoic acid was metabolized in experiments lasting for one year of incubation (206). In multi-substituted compounds, the *meta* substituent is utilized preferentially. Thus, 2,5-dichlorobenzoic acid is reduced to 2-chlorobenzoic acid, 3,4-dichlorobenzoic acid to 4-chlorobenzoic acid, and 2,3,6-trichlorobenzoic acid to 2,6-dichlorobenzoic acid (417). In these experiments, persistence was not correlated with the number of halogens present on the molecule.

Although benzoic acid was always an intermediate in anaerobic mineralization of the chlorobenzoic acids, acclimation to the chlorinated substrate did not result in acclimation to benzoic acid (206). This phenomenon was explored further with the consortium acclimated to 3-chlorobenzoic acid. Metabolism of 3,5-dichlorobenzoic acid to 3-chlorobenzoic acid proceeded with accumulation of 3-chlorobenzoic acid until the parent substrate concentration fell to a low level. Only then was 3-chlorobenzoic acid metabolized to benzoic acid followed by production of methane and CO_2. Similarly, 4-amino-3,5-dichlorobenzoic acid was metabolized to 4-amino-3-chlorobenzoic acid which accumulated until the concentration of parent substrate decreased to a low level, after which the intermediate metabolite was converted to 4-aminobenzoic acid which was not degraded further. These events were postulated to be due to competitive substrate inhibition, in which one enzyme involved in multiple steps of a degradative pathway acts only on the parent compound until its concentration falls below a threshold level (418). Under these conditions, bacteria from environments receiving several structurally related chemicals may metabolize substrates selectively due to competitive substrate inhibition.

The consortium could be acclimated to degrade 4-amino-3,5-dichlorobenzoic acid to its metabolites, even though this substrate was not used as a sole carbon and energy source and was not mineralized (206). Aliquots of the substrate added after acclimation were metabolized without a lag. Partial dechlorination of several compounds by sewage microflora acclimated to nonchlorinated products was reported (221). Substrates attacked include 2-chlorotoluene, 3-chlorobenzoic acid, 4-chloro-2,5-dinitrobenzoic acid, 4-chloroaniline, 4-chlorobiphenyl, 4-chlororesorcinol and 4-chlorobenzonitrile. From 2-47% of the chlorine from these substrates was removed within 2 days. The substrate 4-chloro-3,5-dinitrobenzoic acid was 13 to 45% dechlorinated in sewage but was only 13 to 20% dechlorinated within 20 days by single isolates of bacteria from the sewage.

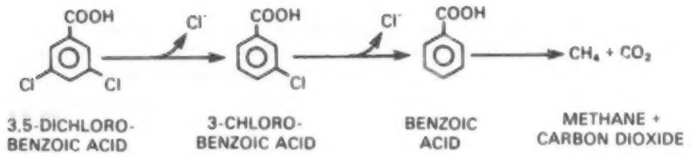

COOH
Cl⁻
COOH
Cl⁻
COOH
Cl
Cl
Cl
CH₄ + CO₂
3,5-DICHLORO-BENZOIC ACID
3-CHLORO-BENZOIC ACID
BENZOIC ACID
METHANE + CARBON DIOXIDE

Figure 37. Representative pathway for the reductive dechlorination of chlorobenzoic acids by anaerobic microbial consortia.

Adapted from References 206, 417.

Reductive dechlorination of pentachlorophenol has been demonstrated in anaerobic soils (217, 266, 320). Resultant products include isomers of tetrachlorophenols, trichlorophenols, dichlorophenols and 3-chlorophenol. The methylated chloroanisole analogues of these isomers have been detected as well. These investigations led to the conclusion that the chloride ions *ortho* and *para* to the hydroxyl group are utilized preferentially (217). Pentachlorophenol metabolism is discussed further in a later section. In contrast, the reductive dechlorination of DDT to DDD described in another section involves the alkyl chlorides but not the chlorides attached to the ring (107, 189a, 202, 234). This has been demonstrated in yeasts as well as bacteria and in pure cultures as well as consortia. Lindane (gamma-hexachlorocyclohexane) is a nonaromatic molecule which is also reductively dechlorinated (Figure 38). The major metabolite is gamma-tetrachlorocyclohexene, followed by benzene, monochlorobenzene, and small amounts of tri-and tetrachlorobenzenes (192).

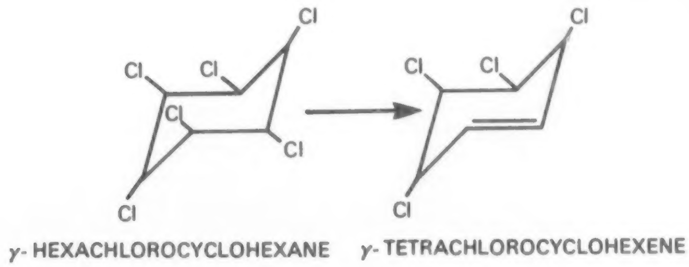

Cl
Cl
Cl
Cl
Cl
Cl
Cl
Cl
Cl
Cl
γ- HEXACHLOROCYCLOHEXANE
γ- TETRACHLOROCYCLOHEXENE

Figure 38. Primary metabolic reductive dechlorination of γ-hexachlorocyclohexane by anaerobic microorganisms.

Adapted from References 192, 267.

SUMMARY

The chlorobenzoic acids can be metabolized to several different intermediate products in bacteria. The most common mechanism results in the conversion of the chlorobenzoic acids to chlorocatechols. If the *meta* cleavage pathway is the only route induced in the bacteria, then chlorocatechols are metabolized usually to chloromuconic semialdehydes, which are not metabolized further. In addition, the *meta* cleavage product of 3-chlorocatechol inactivates the *meta* pyrocatechase, causing an accumulation of the toxic chlorocatechols. This pathway does not lead to mineralization of the chlorinated substrate, and ultimately results in cell death and release of chlorinated intermediates into the environment. In contrast, *ortho* cleavage of chlorocatechols is a successful pathway for chlorobenzoic acid mineralization. The *ortho* pyrocatechase results in the formation of chlorinated muconic acids, which are cycloisomerized with coincident or subsequent elimination of the chloride ion. After release of the chloride ion, the compound is fully metabolized by established cellular mechanisms.

One block to utilization of chlorinated aromatic compounds in organisms expressing the *ortho* pyrocatechase is specificity of the enzyme required for the first oxygenation step. The hybrid strain constructed from *Pseudomonas* sp. B13 and *P. putida* mt-2 incorporates the relatively nonspecific oxygenase of *P. putida* mt-2, carried on the TOL plasmid, into *Pseudomonas* sp. B13 which metabolizes chlorinated compounds via the *ortho* pathway. The hybrid strain is capable of mineralizing a wider range of chlorobenzoic acids than either parent.

Another pathway which has been discovered replaces the chloride ion by a hydroxyl group directly, resulting in a nonchlorinated hydroxybenzoic acid which can be metabolized by established cellular mechanisms. A third series of metabolic pathways results in hydroxylation of the chlorobenzoic acid without loss of prior substituents. A specific and unique enzyme opens the ring and an enzyme has been identified which specifically dechlorinated the resulting aliphatic acid molecule. Data on algal and fungal pathways for metabolism of chlorobenzoic acids are lacking. The relaxed substrate specificities of fungi suggest that these organisms may be of prime importance in metabolism of the chlorobenzoic acids.

SECTION 8
CHLOROBENZENES

MICROBIAL METABOLISM OF CHLOROBENZENES

Chlorobenzenes are used as industrial solvents and diluents for polychlorinated biphenyl compounds (PCBs) and thus have a complementary distribution as pollutants in the environment, including capacitor and transformer storage and disposal (295). They are solvents for paints and appear as byproducts in the textile dyeing industry and in other industries. Pentachloronitrobenzene is used as a fungicide for seeds and soils.

A chemostat seeded with a mixture of soil and sewage samples was used to enrich for an organism capable of utilizing chlorobenzene as a sole growth substrate (366). After nine months an unidentified bacterium, strain WR1306, was isolated which degrades chlorobenzene with stoichiometric release of chloride ion. Detection of the enzymes *cis*-1,2-dihydroxycyclohexa-3,5-diene (NAD+)-oxidoreductase, catechol 1,2-dioxygenase, muconate cycloisomerase, 4-carboxymethylenebut-2-en-4-olide hydrolase and NADH-dependent maleylacetate reductase, and isolation of the metabolite 3-chlorocatechol, enabled construction of a pathway for chlorobenzene dissimilation (Figure 39). The proposed pathway is similar to that demonstrated for the metabolism of other nonphenolic benzene compounds such as 3-chlorobenzoic acid. Chlorobenzene is converted to 3-chlorocatechol which is cleaved by the *ortho* pathway. The substituted muconic acid thus formed is cycloisomerized with coincident or subsequent elimination of chloride. The nonchlorinated intermediate is metabolized to 3-oxoadipic acid which enters the cell's tricarboxylic acid cycle.

Attempts to find other strains of bacteria capable of using chlorobenzene as a sole source of carbon and energy for growth have been hampered by the lipophilicity of the compound (366). Strain WR1306, although capable of growth on chlorobenzene, was inhibited by high concentrations of the compound. Accumulation of the toxic metabolite 3-chlorocatechol is lethal for cells which have not evolved a mechanism for efficient metabolism of this compound. The mechanism of catechol cleavage must be by the *ortho* rather than the *meta* pathway for production of metabolites useful in cell biosynthesis (discussed further in section on chlorobenzoates).

Resting cells of *P. putida* grown on toluene oxidize chlorobenzene and to a lesser extent all three isomers of chlorotoluene (182). Cometabolic growth of *P. putida* on toluene and chlorobenzene results in formation of 3-chlorocatechol. Cells grown on toluene and 4-chlorotoluene metabolize the latter through *cis*-4-chloro-2,3-dihydroxy-1-methylcyclohexa-4,6-diene to 4-chloro-2,3-dihydroxy-1-methylbenzene.

Bacteria were isolated which utilize 1-chloronaphthalene for growth (451a). Chloronaphthalene diol (8-chloro-1,2-dihydro-1,2-dihydroxynaphthalene) and 3-chlorosalicylic acid were recovered as metabolites.

An alternative pathway has been developed which proposes chlorophenols as intermediates in the degradation of chlorobenzenes (19). However, the formation of phenolic products from the dihydrodiol metabolite may occur spontaneously under

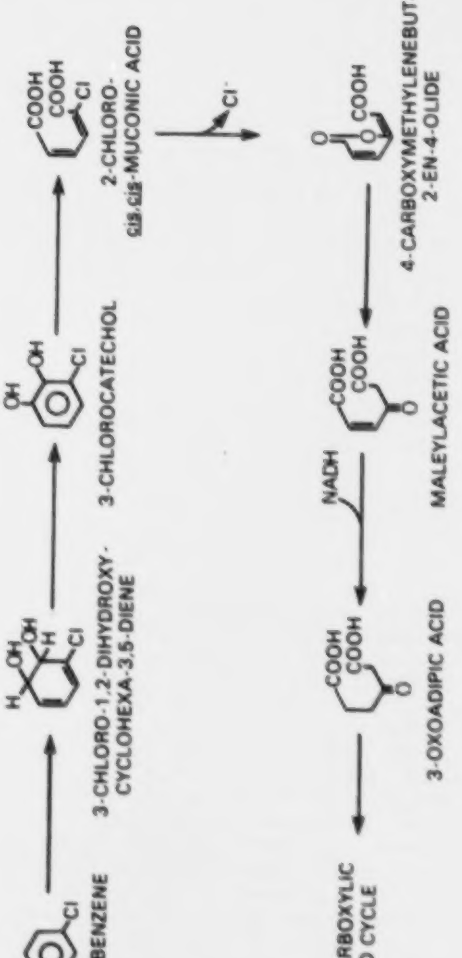

CHLOROBENZENE
3-CHLORO-1,2-DIHYDROXY-CYCLOHEXA-3,5-DIENE
3-CHLOROCATECHOL
2-CHLORO-cis,cis-MUCONIC ACID
Cl⁻
4-CARBOXYMETHYLENEBUT-2-EN-4-OLIDE
MALEYLACETIC ACID
NADH
3-OXOADIPIC ACID
TRICARBOXYLIC ACID CYCLE

Figure 39. Pathway of chlorobenzene mineralization by bacterial strain WR1306. Adapted from Reference 366.

mild acid conditions as well as enzymatically (366). Confirmation of this pathway requires isolation of the enzymes involved.

Pure cultures of bacteria isolated from pond water and pond sediment samples metabolize dichlobenil (2,6-dichlorobenzonitrile) to 2,6-dichlorobenzamide, 2,6-dichlorobenzoic acid and several other metabolites which appeared in trace quantities (131, 132). An *Arthrobacter* sp. which was grown on benzonitrile metabolized more than 70% of ^{14}C-dichlobenil to 2,6-dichlorobenzamide and an additional 20% to other metabolites within 6 days (314).

The degradation of chloronitrobenzenes by fungi has been studied and the mechanism is believed to result in detoxification of the fungicides (94). The yeast *Rhodosporidium* sp., when grown in a complex nutrient medium containing 4-chloronitrobenzene, produces several metabolites. This information enabled a branched pathway to be proposed (Figure 40) (94). The common early steps of 4-chloronitrobenzene metabolism involve sequential reduction of the substrate to form 4-chloronitrosobenzene and subsequently 4-chlorophenylhydroxylamine. This product may be metabolized by two mechanisms. The main pathway is further reduction of the hydroxylamine to 4-chloroaniline, followed by acetylation to produce 4-chloroacetanilide, the major metabolite. This product accumulates in the culture medium. An alternative mechanism involves a shift of the hydroxyl group from nitrogen to carbon (called a Bamberger rearrangement) resulting in conversion of the hydroxylamine to 4-hydroxyaniline and 2-amino-5-chlorophenol. Formation of 4-hydroxyaniline involves loss of the chloride ion and subsequent acetylation results in formation of 4-hydroxyacetanilide.

Pentachloronitrobenzene is metabolized to pentachloroaniline by *Streptomyces aureofaciens, Rhizoctonia solani, Fusarium oxysporum* and many other genera of fungi and actinomycetes (80, 322). In addition, *F. oxysporum* also metabolizes the substrate to pentachlorothioanisole (Figure 41) (322). The introduction of a methylthio group was also noted in the metabolism of 2,4-dichloro-1-nitrobenzene by *Mucor javanicus* AHU6010 (Figure 41) (428). The source of the sulfur atom has not been established. The metabolite may be formed by secondary degradation of a glutathione degradation product, as proposed for rhesus monkey metabolism, or the methylthio group may be transferred from S-adenosylmethionine as demonstrated by cells of *Mycobacterium* sp. (428).

Metabolism of 1,4-dichloro-2,5-dimethoxybenzene (chloroneb) to 2,5-dichloro-4-methoxyphenol by *R. solani* is a detoxification mechanism which results in tolerance by the organism to at least twice the concentration of metabolite as product (204). This conversion occurred only at a high ratio of mycelia to growth medium. *Sclerotium rolfsii* and *Saccharomyces pastorianus* did not metabolize chloroneb. *Neurospora crassa* converts chloroneb to an unidentified product.

In a broad study of 23 species, conversion of chloroneb to 2,5-dichloro-4-methoxyphenol occurred only in cultures actively growing in nutrient medium (468). The most active species was a *Fusarium* sp. which demethylated 60 to 80% of a 5 ppm solution within 10 days. Thirteen other species demethylated the fungicide as well, although *R. solani* neither grew in the presence of nor demethylated chloroneb. *Fusarium* sp. also converted the metabolite back to chloroneb at the rate of 4% in 7 days. Eleven other species performed the same transformation. Demethylation of 2,5-dichloro-4-methoxyphenol to form 2,5-dichlorohydroquinone occurred in four species, but at concentrations of 10% or less of the applied 2,5-dichloro-4-methoxyphenol.

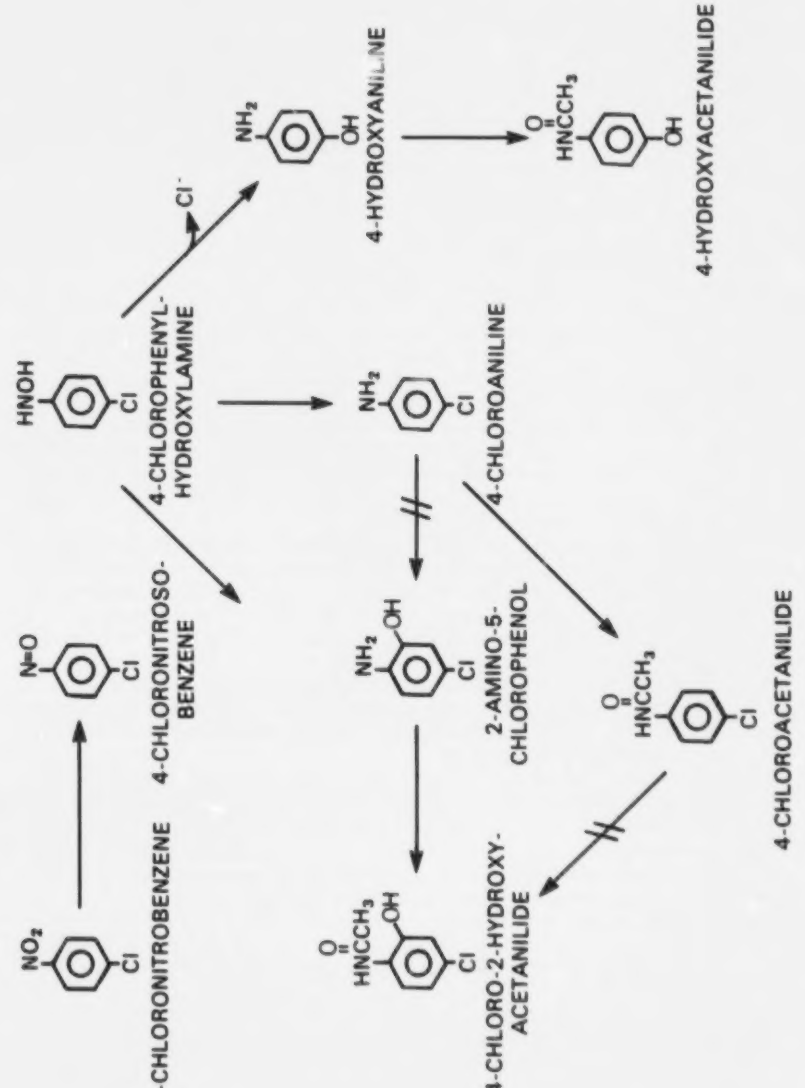

4-CHLORONITROBENZENE
NO2
Cl
N=O
4-CHLORONITROSO-BENZENE
HNOH
4-CHLOROPHENYL-HYDROXYLAMINE
Cl⁻
NH2
OH
4-HYDROXYANILINE
HNCCH3
O
4-HYDROXYACETANILIDE
4-CHLOROANILINE
2-AMINO-5-CHLOROPHENOL
4-CHLOROACETANILIDE
4-CHLORO-2-HYDROXY-ACETANILIDE

Figure 40. Pathways of metabolism of 4-chloronitrobenzene by *Rhodosporidium* sp. Adapted from Reference 94.

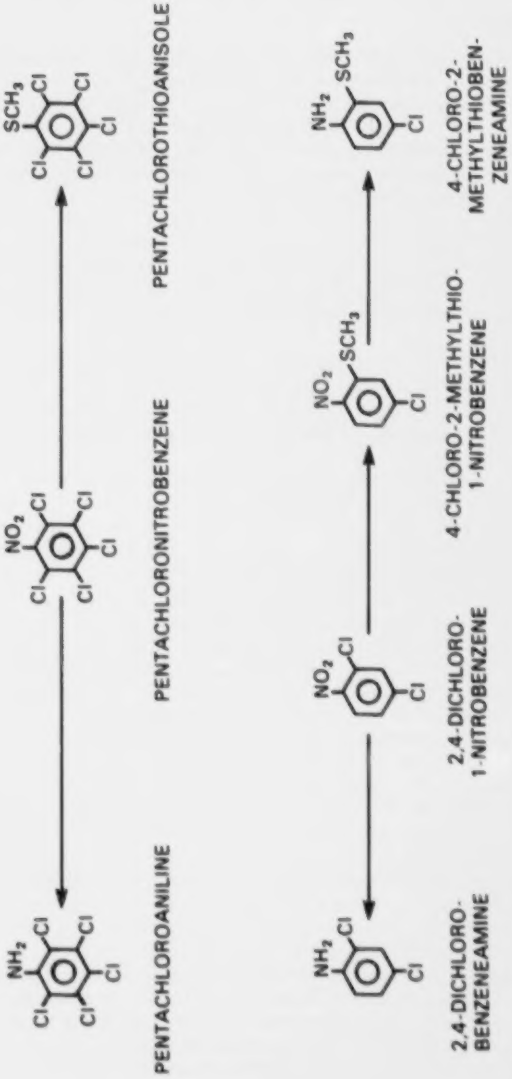

PENTACHLOROANILINE
PENTACHLORONITROBENZENE
PENTACHLOROTHIOANISOLE
2,4-DICHLORO-BENZENEAMINE
2,4-DICHLORO-1-NITROBENZENE
4-CHLORO-2-METHYLTHIO-1-NITROBENZENE
4-CHLORO-2-METHYLTHIOBENZENEAMINE
NH_2
NO_2
SCH_3
Cl

Figure 41. Metabolism of pentachloronitrobenzene by *F. oxysporum* and 2,4-dichloro-1-nitrobenzene by *M. javanicus* AHU6010.

Adapted from References 322, 428.

METABOLISM OF CHLOROBENZENES BY MICROBIAL COMMUNITIES

The chlorobenzenes are largely volatile and attempts to study their biodegradation have been hampered by disappearance of the substrates. A mesocosm experiment using tanks of seawater amended with mixtures of volatile organic compounds showed that at 3 to 7°C both 1,4-dichlorobenzene and 1,2,4-trichlorobenzene disappeared at rates explainable by volatilization (446). At warmer temperatures (20 to 22°C), the rate of disappearance was much more rapid, indicating biodegradation by the planktonic and microbial communities.

Application of 1,2,3- and 1,2,4-trichlorobenzene at a rate of 50 mg/g soil resulted in $^{14}CO_2$ evolution of more than 10% after several weeks (295). Addition of high levels of organic matter increased only 1,2,3-trichlorobenzene mineralization. Extracts of soil dosed with 1,2,3-trichlorobenzene yielded 2,3- and 2,6-dichlorophenol, and 3,4,5-trichlorophenol, while 1,2,4-trichlorobenzene samples yielded 2,4-, 2,5-, and 3,4-dichlorophenol.

A mixed population of soil bacteria precultured on benzene metabolized benzene and chlorobenzenes (20 to 200 mg/l) to chlorophenols (19). Mono- through tetrachlorobenzenes were monohydroxylated at a position *ortho* to the chloride. No phenol was detected in media containing pentachlorobenzene. Diphenyls eventually were detected in the media.

A granular activated carbon column was seeded with a mixed culture of bacteria (primary sewage) and supplied with acetate as a carbon source (52). A biofilm was formed which after acclimation metabolized more than 90% of a 10 to 30 mg/l solution each of chlorobenzene, 1,2-di-, 1,4-di-, and 1,2,4-trichlorobenzene. Partial disappearance of 1,3-dichlorobenzene was noted. Studies with ^{14}C-1,4-dichlorobenzene confirmed that these substrates were mineralized to $^{14}CO_2$.

Hexachlorobenzene was applied to zoysia plots at a rate resulting in 6 ppm in the upper 2 cm of the soil (28). The bulk of the material was volatilized with 24% remaining after 29 days and 3.4% after 19 months. The remaining material was unchanged substrate. The original application resulted in 0.11 ppm in the 2 to 4 cm layer which did not change during the course of the experiment.

Several experiments concerning the metabolism of dichlobenil in soils have shown that the major metabolite is 2,6-dichlorobenzamide (88, 314, 314a, 442a). Other metabolites appeared in trace quantities and could not be identified. After 61 days' incubation of the substrate ^{14}C-labeled in the nitrile group, only trace amounts of $^{14}CO_2$ or volatile ^{14}C-compounds could be recovered. More than 85% of the substrate added at 1 ppm remained unaltered.

Formation of 2,6-dichlorobenzamide in a pond water and sediment system was followed by a decrease in its concentration, indicating further transformation (314). Carbonyl-^{14}C-labeled 2,6-dichlorobenzamide was metabolized with 5.6% converted to $^{14}CO_2$ after 40 days and 28% recovered as metabolites.

Application of 1 mg/l dichlobenil to a farm pond resulted in initial sorption of the herbicide to the soil with subsequent disappearance from both water and soil (371). Less than 10% remained 90 days after treatment. Soils which had been pretreated with 2,6-dichloro-4-nitroaniline showed evolution of $^{14}CO_2$ when treated with this radiolabeled fungicide (189). No $^{14}CO_2$ evolution was noted in soils which had not been pretreated. Some unidentified metabolites were also seen. A pure culture of rod shaped bacteria was isolated which also converted the fungicide to $^{14}CO_2$.

Greenhouse soils treated with pentachloronitrobenzene were analyzed for the presence of metabolites (108). Products recovered included pentachloroaniline, pentachlorothioanisole, and tetrachloronitrobenzene. Hexachlorobenzene and pentachlorobenzene were detected but are known to be present as impurities in technical grade pentachloronitrobenzene.

Extracts of soils amended with 1,000 ppm pentachloronitrobenzene revealed the presence of pentachloroaniline but no polychloroazobenzenes (62). Soils treated periodically for 11 years still showed residual pentachloronitrobenzene and the technical grade impurities tetrachloronitrobenzene, pentachlorobenzene, hexachlorobenzene, pentachloroaniline and methylthiopentachlorobenzene when analyzed 1 to 5 years later (29).

Anaerobic flooded or moist Hagerstown silty clay loam was treated with 10 ppm pentachloronitrobenzene (319). After 40 days' incubation there was no evolution of $^{14}CO_2$ and only slight volatilization of the unchanged substrate. The main metabolite formed was pentachloroanisole, and lesser amounts of pentachlorothioanisole and pentachlorophenol were also detected.

Chloroneb (^{14}C-ring labeled) was applied to soil plots at the rate of 2 lb/acre (370). Another layer of soil was applied to the plots. After 12 months 40% of the original activity was recovered, of which 90% was unchanged substrate and 10% an unidentified metabolite.

SUMMARY

Chlorobenzenes containing less than five chlorines can be mineralized by acclimated populations under permissive conditions. High concentrations of these compounds are toxic to the bacteria. Most of the information regarding metabolism of the chlorobenzenes has come from studies with soil or mixed culture consortia. There is little information available on pathways of metabolism by pure cultures.

A single study indicates that hexachlorobenzene is not metabolized in soils. Pentachlorobenzene was not oxidized in a sole study, although under anaerobic conditions pentachloronitrobenzene is converted to several metabolites.

The available evidence indicates that chlorocatechols or chlorophenols are the primary degradation products of chlorobenzenes. These metabolites can be metabolized by mechanisms discussed in the appropriate sections.

SECTION 9

CHLOROPHENOLS

The chlorophenols are used extensively as antifungal agents and are often applied as a preservative to freshly sawn lumber. They have found some use as herbicides and in food processing plants to control mold (99). Chlorophenols are also common degradation products of chlorophenoxy herbicides. The wood shavings from lumber processes have been used for litter in chicken houses and contain high levels of these chlorophenols, especially 2,3,4,6-tetrachlorophenol and pentachlorophenol (101). The chlorophenols degrade to volatile chloroanisoles via methylation of the oxygen atom and the resulting compounds have been implicated in the "musty taint" of chicken eggs and meat (101).

BACTERIAL METABOLISM OF CHLOROPHENOLS

Studies with *Arthrobacter* spp. have confirmed methylation as a mechanism for chlorophenol metabolism. Conversion of 2,4,6-trichlorophenol to 2,4,6-trichloroanisole has been demonstrated. Methylation is also the predominant reaction in the conversion of guaiacols (o-methoxyphenol) to veratroles (1,2-dimethoxybenzene) by *Arthrobacter* spp. (325). For example, 4-chloroguaiacol, 4,5-dichloroguaiacol, and 3,4,5-trichloroguaiacol are converted to the corresponding veratroles by dense cell suspensions of cultures grown on hydroxybenzoic acid (Figure 42). Low concentrations of catechols have also been found in the culture medium. An exception to this mechanism has been demonstrated in the metabolism by *Arthrobacter* sp. strain 1395 of 3,4,5-trichloroguaiacol to 3,4,5-trichlorosyringol, which requires hydroxylation and subsequent methylation of the previously unsubstituted carbon-6 (Figure 42). This latter compound is resistant to further degradation by this species.

An alternative mechanism results in the formation of chlorocatechols from chlorophenols. Cells of a *Nocardia* sp. grown on phenol metabolize 2-chlorophenol to 3-chlorocatechol, 3-chlorophenol to 4-chlorocatechol, and 4-chlorophenol to 4-chlorocatechol (406). Similarly, phenol-grown *Pseudomonas* sp. B13 or *Alcaligenes eutrophus* cells metabolize 2-chlorophenol to 3-chlorocatechol and 4-chlorophenol to 4-chlorocatechol (262). *Pseudomonas* sp. B13 can utilize 4-chlorophenol as the sole source of carbon and energy, and with this substrate can cometabolize 2-chlorophenol and 3-chlorophenol completely without accumulation of metabolites (262). A phenylcarbamate-degrading *Arthrobacter* sp. also metabolizes 4-chlorophenol to 4-chlorocatechol (466a). While the same initial enzyme is used for the first step in both phenol and 4-chlorophenol degradation, phenol grown cells contain a muconate-lactonizing enzyme which has little activity for 3-chloromuconic acid, the metabolite of 4-chlorophenol (118).

Resting cell suspensions of *Achromobacter* sp. metabolize 4-chlorocatechol to 4-chloro-2-hydroxymuconic semialdehyde and 3,5-dichlorocatechol to 3,5-dichloro-2-hydroxymuconic semialdehyde via a unique catechol 1,6-oxygenase which differs from the more common catechol 2,3-oxygenase (Figure 43) (209). Neither product is metabolized further. *Pseudomonas putida* metabolizes 4-chlorophenol to 4-chlorocatechol, and then employs the *meta* cleavage enzyme catechol 2,3-dioxygenase to

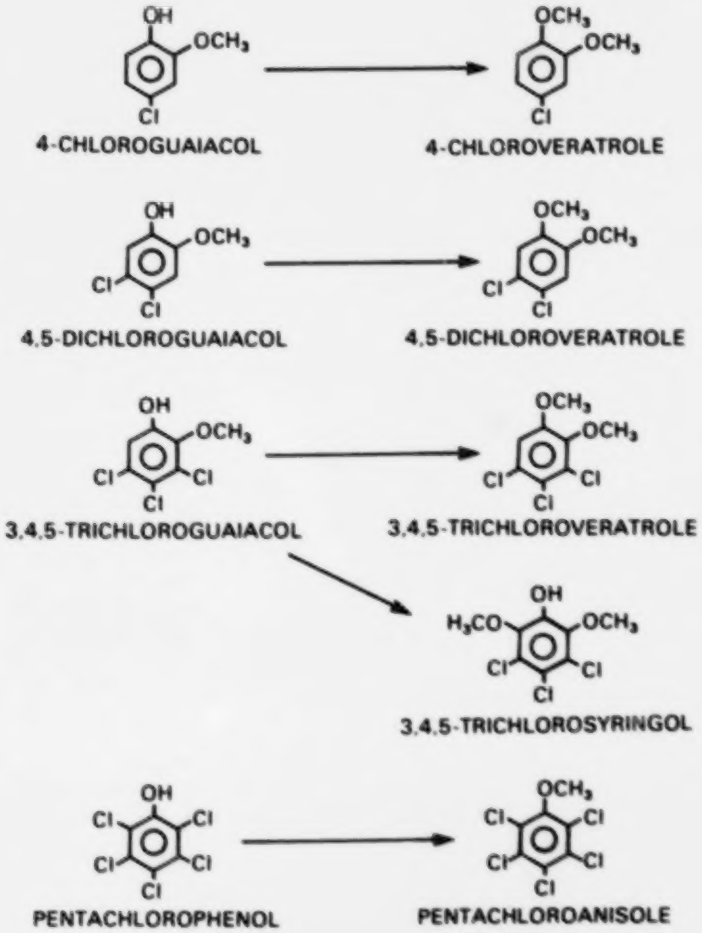
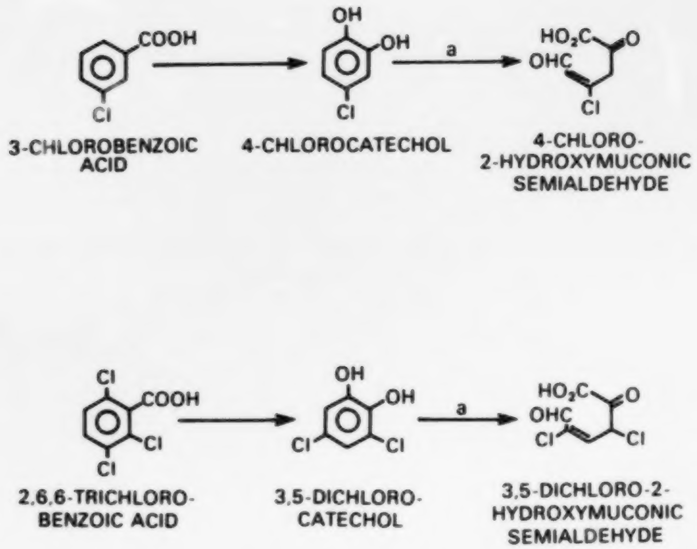

OH
OCH₃
Cl
4-CHLOROGUAIACOL
4-CHLOROVERATROLE
4,5-DICHLOROGUAIACOL
4,5-DICHLOROVERATROLE
3,4,5-TRICHLOROGUAIACOL
3,4,5-TRICHLOROVERATROLE
H₃CO
3,4,5-TRICHLOROSYRINGOL
PENTACHLOROPHENOL
PENTACHLOROANISOLE

Figure 42. Methylation of chlorophenols by *Arthrobacter* spp.
Adapted from Reference 325.


COOH
Cl
3-CHLOROBENZOIC ACID
OH
OH
Cl
4-CHLOROCATECHOL
a
HO_2C
O
OHC
Cl
4-CHLORO-2-HYDROXYMUCONIC SEMIALDEHYDE
Cl
COOH
Cl
Cl
2,6,6-TRICHLOROBENZOIC ACID
OH
OH
Cl
Cl
3,5-DICHLOROCATECHOL
a
HO_2C
O
OHC
Cl
Cl
3,5-DICHLORO-2-HYDROXYMUCONIC SEMIALDEHYDE

a = catechol 1,6-oxygenase

Figure 43. Cometabolism of chlorocatechols via catechol 1,6-oxygenase by resting cell suspensions of *Achromobacter* sp.
Adapted from Reference 209.

produce 2-hydroxy-5-chloromuconic semialdehyde, which accumulates to 10% of the starting substrate. Free chloride amounting to 85% of the substrate is recovered, although a pathway for liberation of the chloride has not been elucidated (333).

Two species of bacteria were utilized to produce a genetically constructed strain with altered ability to metabolize aromatic compounds (388). *Pseudomonas* sp. B13, with ability to metabolize chlorophenols, and *Alcaligenes* sp. A7, which degrades phenol by the *meta* path and has no activity against chlorophenols, were combined to produce a mutant (designated A7-2) which utilizes phenol by the *ortho* path and also metabolizes 2-, 3-, and 4-chlorophenol as well as 3-chlorobenzoic acid. Three enzymes were isolated, pyrocatechase II and cycloisomerase II, which have high activity for chlorinated substrates, and a third enzyme which functions exclusively in the chloroaromatic pathway to perform a dehalogenating cycloisomerization of chloromuconic acids (Figure 44).

The 2,4,5-trichlorophenoxyacetic acid-degrading strain of *P. cepacia* AC1100 can dechlorinate a wide variety of chlorophenols (237). Resting cell suspensions can dechlorinate within 3 hours at 0.1 mM substrate concentration, the following chlorophenols: 2,3-, 2,4-, and 2,5-dichlorophenol, 2,3,4- and 2,4,5-trichlorophenol, 2,3,4,6- and 2,3,5,6-tetrachlorophenol and pentachlorophenol. The strain has less activity against 2,4,6-trichlorophenol and 2,3,4,5-tetrachlorophenol and metabolizes 2,6- and 3,5-dichlorophenol and 2,3,5-, 2,3,6-, and 3,4,5-trichlorophenol poorly.

METABOLISM OF CHLOROPHENOLS BY FUNGI

Fungal metabolism of chlorophenols often involves methylation in a manner analogous to that of bacteria (172). A study of 116 fungal isolates from chicken house litter revealed that 59% produce 2,3,4,6-tetrachloroanisole from 2,3,4,6-tetrachlorophenol at conversion efficiencies of from 1 to 83%. Flask cultures in this study were sealed and incubated for five days, so the transformation may have occurred under aerobic or anaerobic conditions. The fungi demonstrating this ability include *Aspergillus* spp., *Paecilomyces* spp., *Penicillium* spp., and *Scopulariopsis* spp. (100, 101, 172). Some strains metabolize 2,3,4,6-tetrachlorophenol without formation of the anisole, suggesting an alternate mechanism for chlorophenol metabolism (172). The yeast *Rhodotorula glutinis* grown on phenol converts 3-chlorophenol to 4-chlorocatechol (448).

There is little additional information available on fungal metabolism of chlorophenols, although there is evidence to indicate that a *Penicillium* sp. produces 2,4-dichlorophenol as a natural metabolite (8).

METABOLISM OF CHLOROPHENOLS BY MIXED MICROBIAL CULTURES

Soil populations exhibited enhanced rates of metabolism of 2-chlorophenol after prior acclimation by soil percolation (447). Following an initial decrease in 4-chlorophenol concentration during soil percolation, however, additional applications of that substrate were not metabolized. In other experiments, soil inocula mediated the complete disappearance of 4-chlorophenol within 25 days, although neither 2- nor 3-chlorophenol was degraded during that time (193).

Wastewater sludge supernatant liquid required 14 to 25 days for complete disappearance of 16 mg/l 2-chlorophenol and 3-chlorophenol, although 4-chlorophenol disappeared within 14 days (193). Disappearance of 1 mg/l 2- and 4-chlorophenol was faster in polluted acclimated or nonacclimated river water than in diluted sewage inocula experiments (139). The substrates were not degraded by the sewage microorganisms after 25 days, while less than 15 days was required for diappearance of the substrates from river water.

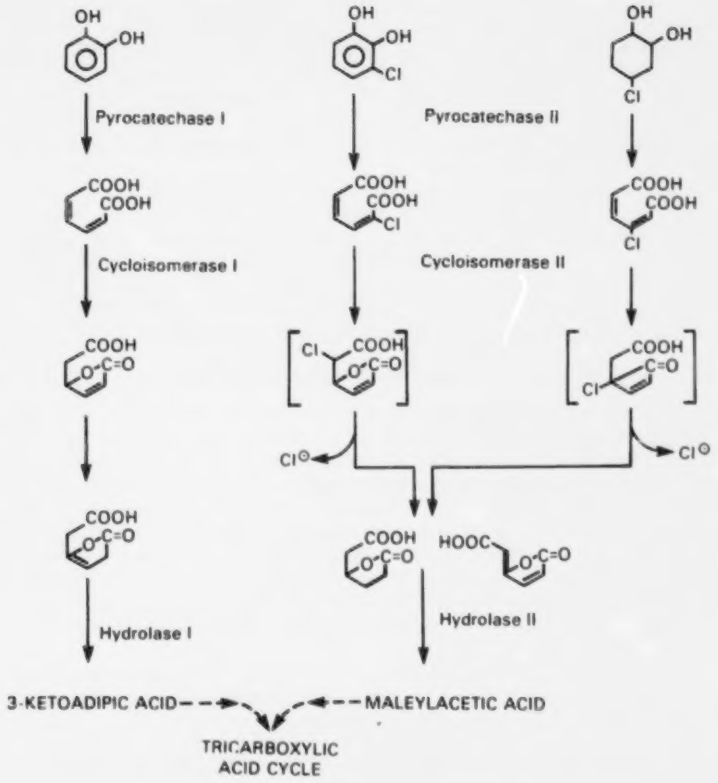

Pyrocatechase I
Pyrocatechase II
Cycloisomerase I
Cycloisomerase II
Hydrolase I
Hydrolase II
3-KETOADIPIC ACID
MALEYLACETIC ACID
TRICARBOXYLIC ACID CYCLE

Figure 44. Action of aromatic and chloroaromatic enzymes from *P. putida* B13 and *P. putida* derivative strains.
Adapted from Reference 261.

An acclimated sludge culture exposed to 100 mg/l substrate was able to metabolize the following compounds within 5 days with chloride release as noted (218): 2-, 3-, or 4-chlorophenol, 100%; 2,4-dichlorophenol, 100%; 2,5-dichlorophenol, 16%; 2,4,6-trichlorophenol, 75%; and sodium pentachlorophenolate, 0%.

The fate of the monochlorophenols when incubated anaerobically with a 10% municipal sewage sludge inoculum was determined (54). At a concentration of 50 mg/l, 2-chlorophenol required 3 weeks, 3-chlorophenol 7 weeks, and 4-chlorophenol 16 weeks for complete disappearance. Mineralization was monitored by measuring net methane production, and results indicated nearly complete mineralization of 2-chlorophenol. Methane was produced from 3-chlorophenol after a lag period of 5 weeks, and 4-chlorophenol was not mineralized. During the degradation of 2-chlorophenol, phenol was recovered as the initial metabolite, followed by methane production. This is consistent with other studies in which dechlorination was shown to be the initial step in the reductive metabolism of 3-chlorobenzoic acids (417).

Fresh undiluted sludge samples also reductively dechlorinated several dichlorophenols with removal of the *ortho* chloride, such that 2,6-dichlorophenol was converted to 2-chlorophenol, 2,3- and 2,5-dichlorophenol to 3-chlorophenol, and 2,4-dichlorophenol to 4-chlorophenol (53). Two isomers which lack an *ortho* substituent, 3,4- and 3,5-dichlorophenol, were not metabolized during the 6 weeks of the experiment.

Undiluted sludge samples were acclimated to the monochlorophenols by repeated inoculations with 20 mg/l substrate, until the cultures could metabolize 25 mg/l within 1 week (53). Each acclimated sludge culture was then challenged with a 20 mg/l solution of other chlorophenols. Cultures acclimated to 2-chlorophenol metabolized both 2- and 4-chlorophenol at equal rates and 2,4-dichlorophenol somewhat more slowly. However, 3-, 2,3-di-, 2,5-di-, and 2,6-dichlorophenol were not metabolized. Sludge inocula acclimated to 3-chlorophenol also metabolized 4-chlorophenol, and 3,4- and 3,5-dichlorophenol but not 2-chlorophenol or 2,3- or 2,5-dichlorophenol. Acclimation to 4-chlorophenol also permitted metabolism of 3-chlorophenol and at much slower rates 2-chlorophenol and 2,4- and 3,4-dichlorophenol as well. The population acclimated to 4-chlorophenol seemed to have a broader substrate range than the other acclimated populations. Incubation of 2- and 4-chlorophenol-acclimated sludge inocula with uniformly ring-^{14}C-labeled 2-and 4-chlorophenol and 2,4-dichlorophenol showed that in all cases nearly complete mineralization of the substrates to ^{14}C-methane and $^{14}CO_2$ occurred.

Experiments in which tainted litter was incubated with sawdust, pentachlorophenol, and 2,3,4,6-tetrachlorophenol, showed nearly quantitative conversion of the latter substrate to 2,3,4,6-tetrachloroanisole (101). There was virtually no conversion in the absence of the litter inoculum. Pentachlorophenol was 50% converted to pentachloroanisole after 29 days.

Aspergillus sydowi, *Scopulariopsis brevicaulis*, and a *Penicillium* sp. were isolated from the litter and each species was also found to be capable of the above substrate conversions.

SUMMARY

Many of the chlorophenol compounds have been shown to be metabolizable by pure cultures or mixed natural populations of microorganisms both aerobically and anaerobically. In most cases complete mineralization occurs. However, the rates of disappearance of the isomers vary widely, depending upon degree of acclimation of the population and other environmental factors. Mixed cultures seem to be required for complete mineralization of the chlorophenols.

SECTION 10
PENTACHLOROPHENOL

Pentachlorophenol (PCP) is widely used in a variety of agricultural and industrial applications as a fungicide, bactericide, insecticide, herbicide, and molluscicide (36, 97, 466). It is most widely used in the United States and elsewhere for wood preservation, both for newly cut timber and for slime control in pulp and paper production. PCP is usually used as a 5% solution in petroleum solvents or as the water-soluble sodium or potassium salt.

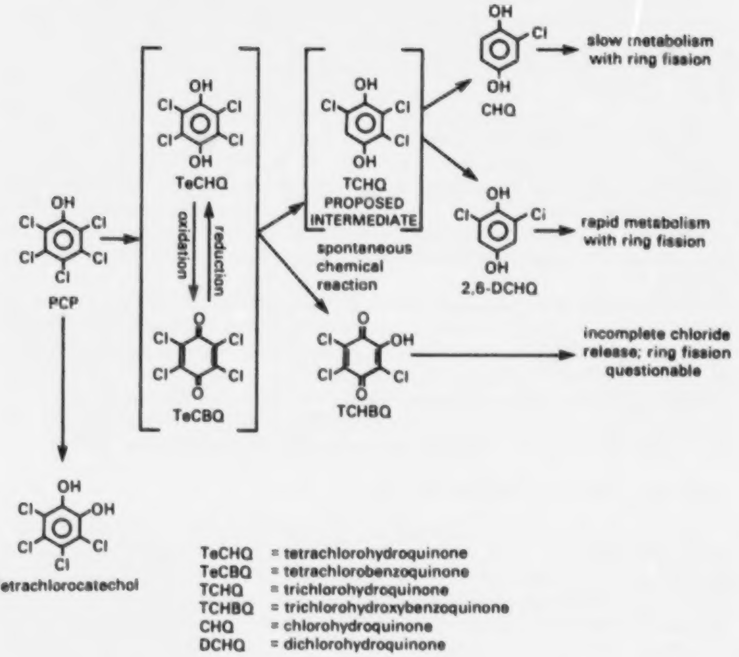
BACTERIAL METABOLISM OF PCP

In spite of its use as a fungicide and bactericide, PCP is metabolizable by a variety of microorganisms. Reports of decomposition of PCP in rice paddy soil and other soils or aquatic environments (90, 217, 253, 266, 282, 320, 354) were followed by experiments with consortia and pure cultures of bacteria which demonstrated chloride release and $^{14}CO_2$ formation from labeled PCP (89, 354, 369, 411, 423, 459). However, few studies have identified metabolites arising from pure culture metabolism of PCP.

Cultures of *Pseudomonas* spp. produce both tetrachlorocatechol and tetrachlorohydroquinone from PCP (Figure 45) (423, 424). These are metabolized rapidly soon after they are produced. There is no evidence of methylation of PCP to form pentachloroanisole. Amino acid analyses with hydrolysates of bacterial cells indicate incorporation of ^{14}C derived from PCP into the cell constituents (423). Pentachlorophenol is metabolized by *Arthrobacter* spp. to pentachloroanisole at levels of less than 0.5% conversion at approximately 44 mM substrate concentration (325).

A bacterium identified as *Mycobacterium* sp. which cannot use PCP as a growth substrate methylates PCP to pentachlaoroanisole (424, 425). Further methylations by washed cell suspensions of this culture result in the formation of tetrachloro-1,2-dimethoxybenzene and tetrachloro-1,4-dimethoxybenzene. Additional metabolites include tetrachlorocatechol, tetrachlorohydroquinone, tetrachloro-2-methoxyphenol and tetrachloro-4-methoxyphenol. The formation of these products indicates that the main metabolite is the methylated derivative of PCP, but in addition, PCP is hydroxylated in the *ortho* or *para* positions and subsequently methylated at these positions. As pentachloroanisole is less toxic to the bacteria than PCP, methylation is suggested as a detoxification mechanism (424). The methylation of PCP was also demonstrated in cell-free systems of *Mycobacterium* sp. (422). The mechanism appears to involve the enzymes that transfer the methyl group from S-adenosylmethionine to the hydroxyl groups of these substrates.

A saprophytic soil corynebacterium was isolated which utilizes PCP as a sole source of carbon and energy for growth (369, 89). By measuring $^{14}CO_2$ evolution, the conversion rate was calculated to be 10 mg PCP per mg of dry cell weight per hour (90). Cells of this isolate, referred to as KC3, when grown on PCP also show immediate uptake, as measured by ultraviolet spectrophotometry, of a wide variety of chlorophenol isomers, including 2,3,5-, 2,3,6-, and 2,4,6-trichlorophenol, 2,3,4,6-, and 2,3,5,6-tetrachlorophenol and pentachlorophenol (89). Uptake of 3,4,5-tri-


OH
Cl
CHQ
slow metabolism with ring fission
TeCHQ
oxidation
reduction
TeCBQ
TCHQ
PROPOSED INTERMEDIATE
spontaneous chemical reaction
2,6-DCHQ
rapid metabolism with ring fission
TCHBQ
incomplete chloride release; ring fission questionable
PCP
Tetrachlorocatechol

TeCHQ = tetrachlorohydroquinone
TeCBQ = tetrachlorobenzoquinone
TCHQ = trichlorohydroquinone
TCHBQ = trichlorohydroxybenzoquinone
CHQ = chlorohydroquinone
DCHQ = dichlorohydroquinone

Figure 45. Proposed pathway for pentachlorophenol (PCP) metabolism by the bacterial culture KC-3 and by *Pseudomonas* sp.
Adapted from References 369, 423.

chlorophenol and 2,3,4,5-tetrachlorophenol is delayed. While the *para* and *meta* monochlorophenols are oxidized, as measured by manometric techniques, the *ortho* isomer is oxidized poorly and phenol itself not at all (89). Chloride is not released to an appreciable degree from any of the monochlorophenols. In general, release of chloride is greatest from the 2,6-substituted di-, tri-, and tetrachlorophenols. Isomers with chloride substitutions in other positions are less well attacked by culture KC3.

Substantial investigations into the metabolism of PCP by the KC3 isolate failed to show accumulation of metabolites in the medium. However, mutants were developed which failed to grow in a PCP-minimal salts medium (369). One of these mutants, designated ER-47, converts PCP primarily to 2,6-dichlorohydroquinone. KC3 parent cells adapted to PCP release chloride from 2,6-dichlorohydroquinone rapidly and without a lag. A trace of monochlorohydroquinone also appears but its role in the pathway of biological degradation is uncertain, as it is only slowly attacked by parent KC3 cells. A second mutant, ER-7, accumulates several metabolites, including tetrachlorohydroquinone, tetrachlorobenzoquinone, and trichlorohydroxybenzoquinone (Figure 36). These three products are converted rapidly and spontaneously from the hydroquinone through the benzoquinone to the more stable hydroxybenzoquinone. The latter product is metabolized by KC3 but dechlorination is not complete and the ring is not ruptured. Tetrachlorohydroquinone is rapidly metabolized to 2,6-dichlorohydroquinone but this metabolic transformation must compete with the rapid spontaneous transformation to the trichlorohydroxybenzoquinone.

Another series of experiments explored the metabolism of sodium pentachlorophenate by a wide variety of bacteria metabolically active for phenols, chlorophenols or chlorobenzenes (379). Metabolites were identified by detecting acetyl derivatives using combined gas chromatography and mass spectrometry. With few exceptions metabolites occurred in concentrations of less than one percent of the starting material. Reported metabolites included PCP-acetate, pentachloroanisole, tetrachloroanisoles, tetrachlorophenols, and tetrachlorodihydroxybenzenes.

FUNGAL METABOLISM OF PCP

The role of fungi in detoxifying PCP has been studied to some degree. Fungi associated with PCP-treated wood reduce PCP to a less toxic metabolite (122, 288). Three *Trichoderma* spp. metabolized sodium pentachlorophenate (Na-PCP) within 2 weeks in a malt extract medium as well as on wood treated with Na-PCP (98). Pentachloroanisole was detected in the culture medium of *T. virgatum* after 5 days' incubation at levels corresponding to 10-20% of the starting Na-PCP. It is unclear whether this is an integral step of the pathway or whether methylation is a side reaction (98).

During a comparison of the growth of several species of fungi on PCP, *Trichoderma* spp. were the only ones which reduced PCP levels after 12 days' incubation at 5 to 10 mg/l concentration (98). Fungi inactive against PCP were *Cephaloascus fragrans*, *C. pilifera*, *Graphium* spp., and *Penicillium* sp. Another experiment showed that *Trichoderma viride* and *Coniophora puteana* reduced the concentration of PCP in treated wood blocks, although *C. puteana* was much more sensitive to PCP in liquid culture (441). It was postulated that the presence of an alternative substrate of wood or the binding capacity of PCP to wood reduced exposure of the fungus to below the toxic level, thus permitting metabolism of PCP.

DISAPPEARANCE OF PCP IN ENVIRONMENTAL SAMPLES

The standard procedure of applying PCP in a carrier solvent to wood products has complicated subsequent analyses of disappearance and biodegradability. A carrier which is too volatile will carry PCP with it as it evaporates (277). A carrier which retains liquidity at ambient temperatures will bleed from the wood until an equilibrium is established. PCP in solution will be carried along, reducing the final concentration in the wood and increasing the amount reaching the surrounding environment.

Extraction and analysis procedures are also subject to error, including incomplete extraction due to poor choice of extracting solvent, and use of procedures which extract pure PCP but not polymerized molecules, which may be present in technical grade PCP at levels as high as 18 percent (277).

Degradation of PCP with release of chloride and CO_2 has been demonstrated in a number of environments. In a waste stream continuously contaminated with PCP, acclimation occurred after 3 weeks (354). The microflora, particularly the attached bacteria, metabolized up to 0.43 ppm influent concentration. Pure cultures were isolated from the waste stream which were capable of mineralizing 100 mg/l PCP in 90 hr with almost complete chloride release.

A soil perfusion apparatus using rice paddy soil effected disappearance of PCP with more than 90% liberation of chloride (459). A *Pseudomonas* sp. isolated from the enrichment culture degraded 40 mg/l PCP in 10 days with complete chloride release.

PCP added to moist garden soil at 150 to 200 mg/l soil-water concentration was 25% metabolized after 12 days when the experiment was conducted using outdoor shaded test plots (128). When a culture of *Arthrobacter* sp. ATCC 33790 was mixed into the soil, about 85% of the PCP disappeared during the same time. Under laboratory conditions addition of the bacterial culture reduced the half-life for PCP disappearance from 12 - 14 days to 1 day. This *Arthrobacter* sp. utilizes PCP as the sole source of carbon and energy with complete release of chloride (130).

Comparisons of aerobic and anaerobic metabolism of PCP have shown that aerobic metabolism is much more efficient (282). Enrichment cultures established in fermentors fed with 2 mg/l PCP revealed a half-life of 0.36 days under aerobic conditions and 192 days under anaerobic conditions. Addition of glucose or 4-chlorophenol as cometabolic substrates depressed the rate of PCP metabolism.

Soils treated with 10 mg/l PCP and incubated for 24 days under aerobic conditions revealed considerable loss of ^{14}C-labeled material from the system (320). Of 59% total recovered material, 51% was identified as pentachloroanisole. Volatile products and CO_2 were not measured. In the same system maintained under anaerobic conditions, 7% of the material was converted to metabolites and no $^{14}CO_2$ was detected. Metabolites included about 5% pentachloroanisole and lesser amounts of 2,3,6-trichlorophenol, 2,3,4,5-tetrachlorophenol and 2,3,5,6-tetrachlorophenol.

PCP applied to flooded paddy soil, simulating anaerobic conditions, was metabolized after 3 weeks to the following products: 3-chlorophenol, 3,4-, and 3,5-dichlorophenol, 2,3,5-, and 2,4,5-trichlorophenol, and 2,3,4,5-, 2,3,4,6-, and 2,3,5,6-tetrachloroanisole (217).

The rate of PCP metabolism in 11 soils was found to be related to the organic matter content of the soils (266). Degradation products included a mixture of tri- and tetrachlorophenols.

A major PCP spill on the Mississippi River Gulf Outlet left PCP levels as high as 1.60 mg/g in the sediment (109). At 18 months there was no detectable PCP in the sediment. Studies arising from the spill indicated that the degradation rates increased with increasing sediment redox potential. Maximum degradation occurred at pH 8 at +500 mV. Less degradation occurred at pH 9 and at pH less than 8.

PCP-degrading bacteria have been isolated both from polluted sites and from sites not known to be contaminated with PCP (411). An enrichment consortium established under continuous culture conditions became adapted to metabolize 500 mg/l PCP. *Arthrobacter* sp. strain NC was isolated from the culture and metabolized 100 mg/l PCP until the pH decreased to 6.15. Upon adjustment to pH 7.1 the residual PCP was metabolized. The strain was capable of growth at pH 6.0 upon other substrates. Other experiments showed a correlation of toxicity with the acid form of PCP.

SUMMARY

In summary, there is evidence that PCP is attacked by bacteria and fungi cometabolically or as sole source for growth with release of chloride and CO_2. The pathway involves dechlorination and hydroxylation either *ortho* or *para* to the phenolic hydroxyl group, forming a catechol or a quinone, respectively. However, the mechanism of this process is not understood and the enzymes involved have not been isolated. Further, the steps of the pathway leading to carbon incorporation into cell contents and CO_2 formation have not been elucidated. In fungi, methylation has been detected as a prominent metabolic process, but its role in PCP degradation has not been established.

SECTION 11
CHLOROPHENOXY AND CHLOROPHENYL HERBICIDES

Compounds with an arylcarboxylic acid parent structure have plant growth-regulating properties. They produce physiological effects such as morphogenic abnormalities, promote the rooting of cuttings, and aid in setting fruit in the absence of pollination (445).

There are three principal chlorine-substituted phenoxyacetic acids used widely as herbicides, (2,4-dichlorophenoxy)acetic acid (2,4-D), (2,4,5-trichlorophenoxy)acetic acid (2,4,5-T), and (4-chloro-2-methylphenoxy)acetic acid (MCPA). They are selective against broadleaved weeds and woody broadleaved plants and are commonly used in lawns, grass pastures, and cereal crops (283). Other phenoxyalkanoic acids are useful in controlling weed species which are resistant to the phenoxyacetic acids. The phenoxybutyric acid herbicides have very low toxicity to plant species such as legumes which are damaged by exposure to phenoxyacetic acids. After application, they are activated by target plants (weeds) which β-oxidize them to their corresponding toxic phenoxyacetic acids. Other herbicides in this class include the phenoxyethyl esters, which are applied when deep soil penetration is required or in noncrop areas.

The phenoxyalkyl acid herbicides are detoxified in soils and aquatic environments due to microbial action (13, 60, 111, 283, 343, 376, 377). Bacteria and fungi which metabolize the herbicides have been isolated from soils (Table 4). The products of microbial metabolism may be phytotoxic or they may result in inactivation of the herbicide (290, 292). These products may be similar to those formed as a consequence of plant metabolism, such as 2,4-dichlorophenol from 2,4-D and 2,4,5-trichlorophenol from 2,4,5-T. There is evidence that 2,4,5-trichlorophenol, rather than 2,4,5-T, is the active agent which damages the plants (283). Some processes of microorganisms prevent activation of the herbicides rather than actually detoxifying them (3). For this reason the pathways by which microorganisms metabolize the phenoxy herbicides are of importance in determining the choice of herbicide for a given application.

2,4-D

The herbicide most extensively studied has been 2,4-D. Bacteria including *Pseudomonas* sp., *Arthrobacter* sp., *Achromobacter* sp., *Mycoplana* sp., and *Flavobacterium peregrinum*, cleave the molecule at the ether linkage between the oxygen and the aliphatic side chain to form glyoxylic acid and 2,4-dichlorophenol (Figure 46) (31, 32, 40, 152, 285, 286, 412, 433, 434, 451). The latter compound is metabolized to 3,5-dichlorocatechol, *cis,cis*-2,4-dichloromuconic acid, 2-chloro-4-carboxymethylene but-2-enolide and 2-chloromaleylacetic acid (46, 393). Chloromaleylacetic acid is degraded further to succinic acid via 2-chloro-4-ketoadipic acid and chlorosuccinic acid. The entire pathway has also been demonstrated using cell-free extracts of *Arthrobacter* sp. (40, 44, 126, 142, 393, 434). Cleavage of the ether-oxygen bond in phenoxyacetic acid to form the phenol has been proven to occur between the aliphatic side chain and the ether-oxygen in experiments with *Arthrobacter* sp. using 1802 (198).

TABLE 4. MICROORGANISMS THAT METABOLIZE PHENOXY ACIDS

Phenoxy acid	Organism	References
2,4-D	*Achromobacter* sp.	31, 32, 412, 414
	Arthrobacter sp.	45, 126, 285, 434, 44, 46, 284, 286
	Arthrobacter globiformis	283
	Corynebacterium sp.	375
	Flavobacterium peregrinum	405a, 411a, 412, 414
	Mycoplana sp.	451
	Nocardia sp.	283
	Pseudomonas sp.	146, 169, 170, 171, 142, 152, 171a
	Sporocytophaga congregata (F. aquatile)	225
	Streptomyces viridochromogenes	51a
2,6-D	*Achromobacter* sp.	31, 32
2-Chlorophenoxyacetic acid	*Achromobacter* sp.	31, 32, 412, 414
	Arthrobacter sp.	45, 284, 285, 286, 44, 46, 125, 434
	F. peregrinum	412, 414
4-Chlorophenoxyacetic acid	*Pseudomonas* sp.	142, 146, 152
	Achromobacter sp.	31, 32, 412, 414
	Arthrobacter sp.	45, 284, 285, 286
	F. peregrinum	412, 414
	Mycoplana sp.	451
	Nocardia sp.	283
	Pseudomonas sp.	126, 142, 143, 146, 152, 169, 170, 171, 171a

(continued)

TABLE 4. (continued)

MCPA	*Achromobacter* sp.	31, 32, 412, 414
	Arthrobacter sp.	45, 284, 285, 286, 44, 46, 126, 434
	F. peregrinum	45
	Mycoplana sp.	451
	Pseudomonas sp.	142, 146, 152, 169, 170, 171, 171a
2,4,5-T	*Achromobacter* sp.	31, 32
	Brevibacterium sp.	207
	Mycoplana sp.	451
	S. viridochromogenes	51a

The enzymes mediating the degradation of 2,4-D are relatively nonspecific. Oxygen and a reduced pyridine nucleotide (NADH or NADPH) are required, indicating that the enzyme(s) may be a mixed function oxidase (44). The broad specificity of the enzymes is reflected in the findings that *Pseudomonas* sp. cells grown on 2,4-D also metabolize 4-hydroxyphenoxyacetic acid and phenoxyacetic acid (285). *Arthrobacter* sp. cell-free extracts grown on 2,4-D also oxidize MCPA, 2,4-dichlorophenol, 4-chloro-2-methylphenol and 3,5-dichlorophenol. Neither 6-hydroxy-2,4-dichlorophenol nor 2,4-dichloroanisole is oxidized, indicating that neither is an intermediate in 2,4-D metabolism (284). The enzyme extracts also convert 2-chlorophenoxyacetic acid to 2-chlorophenol and 4-chlorophenoxyacetic acid to 4-chlorophenol which subsequently forms 4-chlorocatechol. Catechol is converted to *cis,cis*-muconic acid and 4-chlorocatechol to *cis,cis*-3-chloromuconic acid. Chloride is released from 3-chloromuconic acid to form 4-carboxymethylene but-2-enolide, maleylacetic acid and subsequently succinic acid (Figure 47) (40, 44, 434). This pathway is analogous to that demonstrated for 3,5-dichlorocatechol during 2,4-D degradation.

A *Corynebacterium* sp. isolated from soil by enrichment culture metabolized 2,4-D with nearly complete chloride release after 48 hours (375). An application rate to soil of 3,000 ppm was metabolized but neither growth nor metabolism was noted upon application of 3,500 ppm. No metabolites were seen during the incubation period.

A bacterial strain tentatively identified as *F. peregrinum* was isolated from enrichment culture with 2,4-D in soil (414). This strain metabolized 100 ppm 2,4-D in 25 days and upon addition of 0.1% yeast extract metabolized 0.1% 2,4-D in 12 to 16 days. Chloride release was estimated at 70% of that in 2,4-D within 39 days.

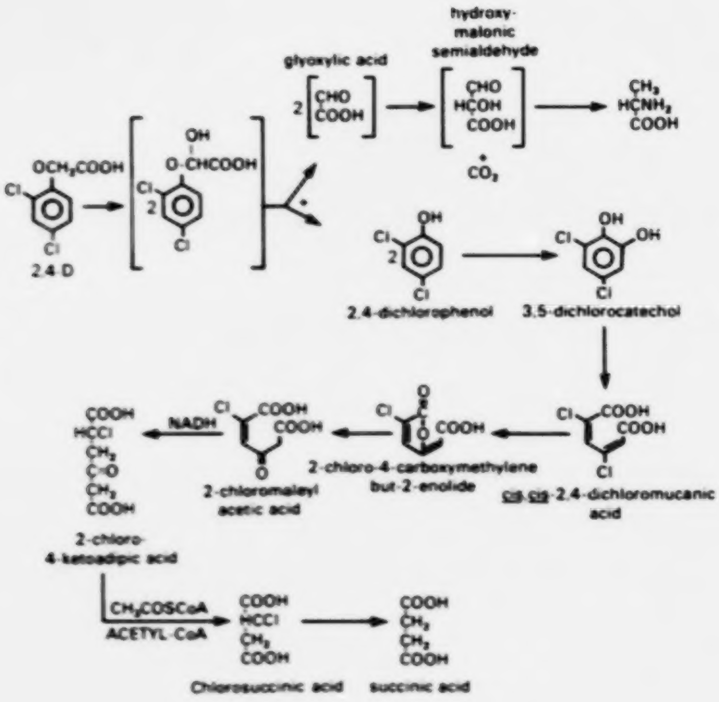

hydroxy-
malonic
semialdehyde
glyoxylic acid
2
CHO
COOH
CHO
HCOH
COOH
CH3
HCNH2
COOH
CO2
OH
OCH2COOH
O-CHCOOH
Cl
2
Cl
2,4-D
OH
OH
2,4-dichlorophenol
3,5-dichlorocatechol
COOH
HCCl
CH2
C=O
CH2
COOH
NADH
COOH
COOH
O
2-chloromaleyl
acetic acid
2-chloro-4-carboxymethylene
but-2-enolide
COOH
cis,cis-2,4-dichloromucanic
acid
2-chloro-
4-ketoadipic acid
CH3COSCoA
ACETYL-CoA
COOH
HCCl
CH2
COOH
COOH
CH2
CH2
COOH
Chlorosuccinic acid
succinic acid

Figure 46 Bacterial metabolism of 2,4-D

Adapted from References 46, 142, 433

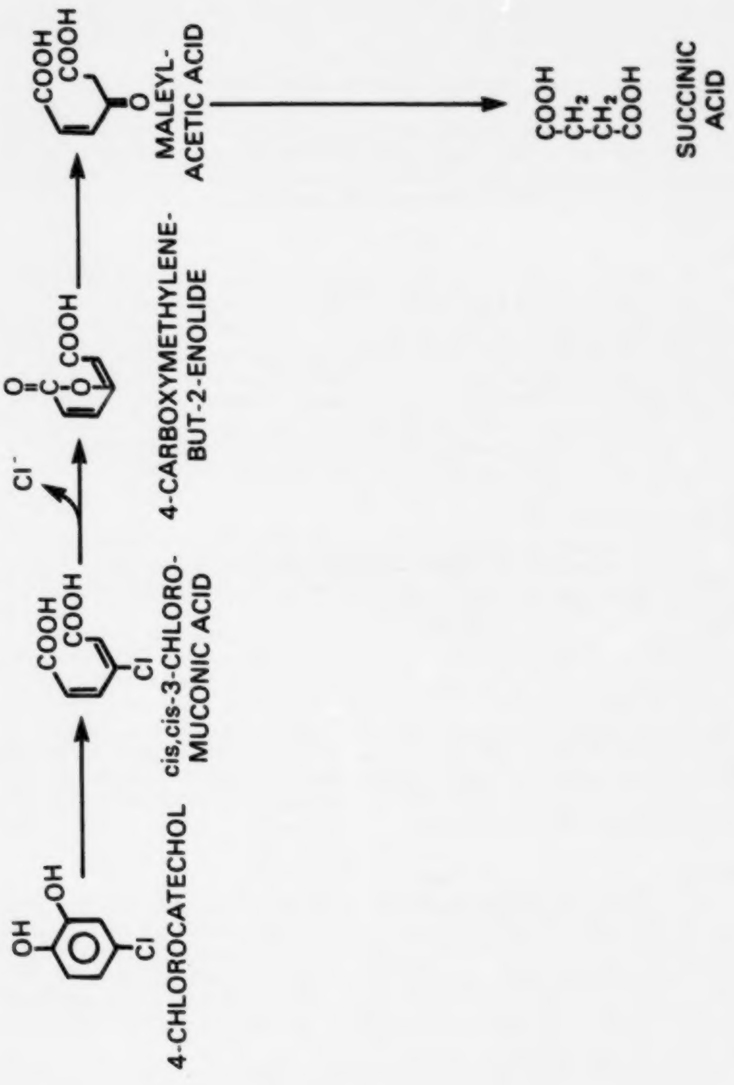

OH
OH
Cl
4-CHLOROCATECHOL
COOH
COOH
Cl
cis,cis-3-CHLORO-MUCONIC ACID
Cl⁻
O=C
O
COOH
4-CARBOXYMETHYLENE-BUT-2-ENOLIDE
COOH
COOH
O
MALEYL-ACETIC ACID
COOH
CH₂
CH₂
COOH
SUCCINIC ACID

Figure 47. Metabolism of 4-chlorocatechol by *Arthrobacter* sp. Adapted from References 46, 434.

Flavobacterium aquatile metabolized 0.01% 2,4-D in sterile soil, nonsterile soil, and on solid agar plates, but not in a soil extract medium or on semisolid agar (225). MCPA was not metabolized in sterile soil. In similar experiments, *Corynebacterium* sp. metabolized both herbicides in sterile soil and on solid agar.

A number of bacteria were isolated by enrichment culture from sewage or soil amended with 2,4-D or 2,4,5-T (376). None utilized either substrate as the sole source of carbon. Forty-one of 52 strains cometabolized 2,4-D only while 19 strains utilized both 2,4-D and 2,4,5-T. Experiments with these 19 isolates incubated with 2,4,5-T in nutrient medium showed that 12 isolates produced chloride ion and 8 produced a phenolic compound with or without concomitant production of free chloride.

MCPA

The metabolism of MCPA has been studied extensively in cultures of *Pseudomonas* sp. NCIB 9340 and *Arthrobacter* sp. (45, 46, 169, 170, 412) as well as cell-free extracts of *Arthrobacter* sp. (40). Initial attack on the molecule results in oxidative cleavage of the ether linkage to form a phenol and glyoxylic acid (Figure 48) (169). The phenol thus formed is 4-chloro-2-methylphenol (5-chloro-o-cresol). In *Pseudomonas* sp. NCIB 9340 this product is metabolized to 5-chloro-3-methylcatechol and then to *cis,cis*-4-chloro-2-methylmuconic acid. The chloride ion is lost upon lactonization by dehydrochlorination to form 4-carboxymethylene-2-methyl-2,3-butenolide and subsequently 4-hydroxy-2-methylmuconic acid (170). Formation of the two double bonds of the lactone is an unusual feature in the metabolism of aromatic compounds by bacteria.

The three enzymes mediating the conversion of 5-chloro-3-methylcatechol to 4-hydroxy-2-methylmuconic acid have been isolated (171). These enzymes, responsible for ring cleavage, lactonization, and delactonization, confirm that lactonization and dehalogenation is a one-step process.

The enzymes which attack MCPA are also relatively nonspecific. Oxygen as well as NADH or NADPH are required for enzymatic activity. *F. peregrinum* cells grown in the presence of MCPA are induced to oxidize 2,4-D as detected by manometric techniques, although MCPA is not metabolized (413). A strain thought to be an *Achromobacter* sp., isolated from enrichment culture with MCPA, metabolized 50 mg/l MCPA or 2,4-D with addition of 0.05% yeast extract in 4 days (414). Experiments showed a faster rate of oxygen uptake with 2,4-D than MCPA although the organism was cultured on MCPA. Nonacclimated cultures showed no oxygen uptake with either MCPA or 2,4-D.

2,4,5-T

The tri-chlorinated phenoxy herbicide, 2,4,5-T, has proven much more difficult to degrade. The utilization of 2,4,5-T by a *Pseudomonas* sp. appears not to be plasmid encoded, unlike 2,4-D and MCPA metabolism (154). The herbicide is metabolized to 2,4,5-trichlorophenol by both *P. fluorescens* and *P. cepacia* AC1100 (249, 377). *Brevibacterium* sp. when grown on benzoic acid converts 2,4,5-T to 3,5-dichlorocatechol without a lag, indicating removal of one chloride ion (207). This latter compound can be metabolized (Figure 46).

Pseudomonas cepacia AC1100 is a strain modified in the laboratory which utilizes 2,4,5-T as the sole source of carbon and energy for growth (249). More than 97% of a 1 g/l solution was degraded within 6 days with stoichiometric chloride release. In 2 hr, resting cells promoted 50% disappearance of 2,4,5-T although only 15% chloride release was evident. Within 24 hours, there was complete substrate disappearance with 94% chloride release. Resting cells also mediated release of more than 80% of the chloride from 2,4,5-trichlorophenol, 2,3,4,6-tetrachlorophenol and pentachloro-

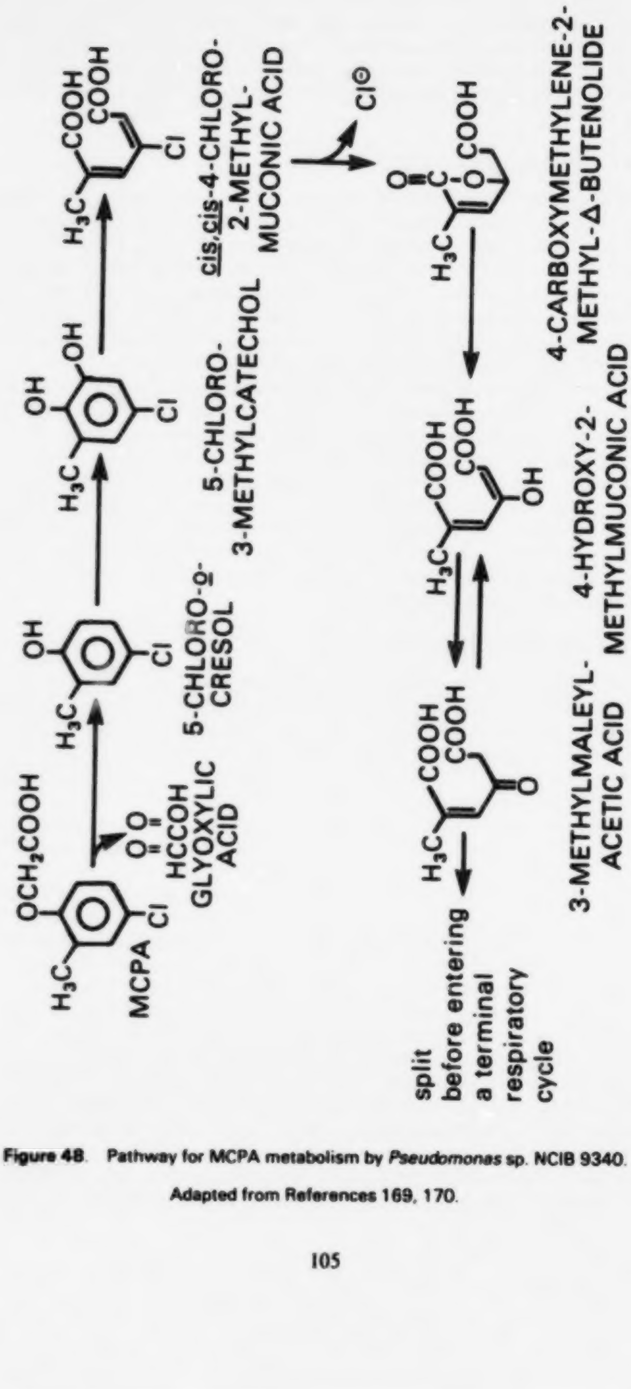

MCPA
OCH_2COOH
HCCOH
GLYOXYLIC ACID
5-CHLORO-o-CRESOL
5-CHLORO-3-METHYLCATECHOL
cis,cis-4-CHLORO-2-METHYL-MUCONIC ACID
Cl⊖
4-CARBOXYMETHYLENE-2-METHYL-Δ-BUTENOLIDE
4-HYDROXY-2-METHYLMUCONIC ACID
3-METHYLMALEYL-ACETIC ACID
split before entering a terminal respiratory cycle

Figure 48. Pathway for MCPA metabolism by *Pseudomonas* sp. NCIB 9340.

Adapted from References 169, 170.

phenol. Additionally, resting cells were also shown by oxygen electrode determination to oxidize 2,4-D at relatively high rates but not (2,4-5-trichloro-phenoxy) propionic acid. Phenoxyacetic acid was oxidized at low rates only. When inoculated into soil *P. cepacia* AC1100 mediated 95% chloride release of a 1 mg/g application of 2,4,5-T within 1 week (85). The optimum conditions were 25% moisture content at 30°C.

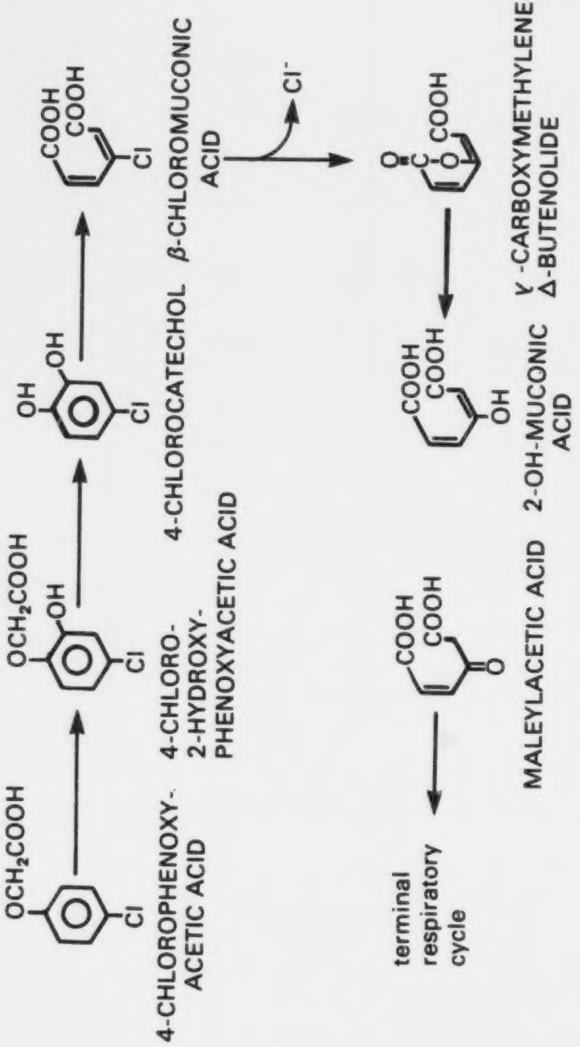
4-CHLOROPHENOXYACETIC ACID

The degradation of 4-chlorophenoxyacetic acid by a soil pseudomonad proceeds through 4-chloro-2-hydroxyphenoxyacetic acid to 4-chlorocatechol (Figure 49) (143). This organism also metabolizes 4-chlorocatechol to *cis,cis*-3-chloromuconic acid. While direct evidence for subsequent steps in the pathway was not obtained, the culture medium did contain a lactone which is analogous to that described for *Arthrobacter* sp. The pseudomonad was not induced to grow on 4-chlorophenol and this product was not found in the culture medium, indicating that this compound is not an intermediate in 4-chlorophenoxyacetic acid metabolism.

An unidentified gram-negative organism isolated from soil also metabolizes 4-chlorophenoxyacetic acid through 4-chloro-2-hydroxyphenoxyacetic acid and 4-chlorocatechol, as determined by simultaneous adaptation experiments (146). The same technique was used to determine that *Achromobacter* sp. grown on 4-chlorophenoxyacetic acid immediately oxidizes 4-chloro-2-hydroxyphenoxyacetic acid, 4-chlorocatechol, and catechol, but not 4-chlorophenol (414). The first step of 4-chlorophenoxyacetic acid metabolism in these pathways is hydroxylation of the ring, followed by ether cleavage. This is in contrast to *Arthrobacter* sp. metabolism of several chlorophenoxy compounds in which cleavage of the ether linkage is the primary step yielding 4-chlorophenol (284).

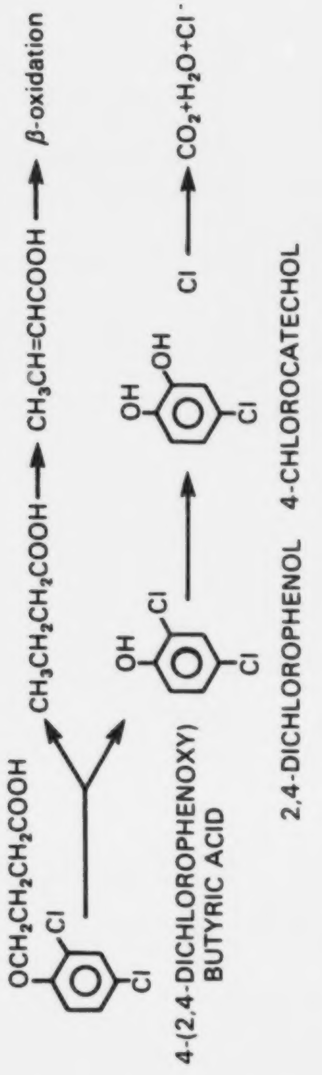
OTHER PHENOXY HERBICIDES

The metabolism of phenoxy herbicides with longer aliphatic side chains has also been studied. Two mechanisms appear to mediate degradation of these compounds. The primary mechanism is β-oxidation, a mechanism common to plants (283). Evidence for β-oxidation comes from observations with cultures of *Flavobacterium* sp. which were grown on 4-(2,4-dichlorophenoxy)butyric acid (4-(2,4-D)B) and then tested for products arising from oxidation of higher carbon-number homologs. Phenols were detected in all cases but more so with compounds containing an odd number of carbons in the side chain (291). When the side chain contained an odd number of carbons, primarily 2,4-dichlorophenol was recovered, while 2,4-D was recovered from metabolism of compounds with an even number of carbons in the side chain. Extracts of these cultures also contained free aliphatic acids which indicates ether cleavage, a second mechanism of phenoxy acid degradation similar to that shown for other herbicides (290, 292). This organism when grown on 4-(2,4-D)B also oxidizes (as determined by manometric techniques) 3-(2,4-D)propionic acid, 4-(4-chlorophenoxy)butyric acid, 4-(4-methyl-2-chlorophenoxy)butyric acid, 2,4-dichlorophenol, and 4-chlorocatechol but not 2,4-D (60, 292). The failure to oxidize 2,4-D argues against β-oxidation as the controlling mechanism for these degradations as the product of β-oxidation of 4-(2,4-D)B would be 2,4-D (292). The enzymes involved in these oxidations are adaptive rather than constitutive. The oxidation of these substrates led to the proposal of a pathway for the degradation of 4(2,4-D)B through 2,4-dichlorophenol to 4-chlorocatechol with loss of one chloride ion and subsequent metabolism of 4-chlorocatechol by established pathways (Figure 50). The side chain is cleaved initially at the ether linkage and then is metabolized by β-oxidation (292).



4-CHLOROPHENOXY-ACETIC ACID
4-CHLORO-2-HYDROXY-PHENOXYACETIC ACID
4-CHLOROCATECHOL
β-CHLOROMUCONIC ACID
Cl⁻
γ-CARBOXYMETHYLENE-Δ-BUTENOLIDE
2-OH-MUCONIC ACID
MALEYLACETIC ACID
terminal respiratory cycle

Figure 49. Pathway for 4-chlorophenoxyacetic acid metabolism by a soil pseudomonad. Adapted from Reference 143.



OCH₂CH₂CH₂COOH
Cl
Cl
4-(2,4-DICHLOROPHENOXY) BUTYRIC ACID
CH₃CH₂CH₂COOH
CH₃CH=CHCOOH
β-oxidation
OH
Cl
Cl
2,4-DICHLOROPHENOL
OH
OH
Cl
4-CHLOROCATECHOL
CO₂+H₂O+Cl⁻

Figure 50. Pathway for the metabolism of 4-(2,4-dichlorophenoxy) butyric acid by *Flavobacterium* sp.

Adapted from Reference 292.

Two species of *Nocardia*, *N. opaca* strain T16 and *Nocardia* sp. strain P2 have been shown to use β-oxidation for degradation of phenoxy acid homologs above the acetic congener (461). The corresponding chlorinated phenols are generated from (3-chlorophenoxy)propionic and (4-chlorophenoxy)propionic acids. The six-carbon homolog (2-chlorophenoxy)caproic acid is metabolized to (2-chlorophenoxy)butyric acid and (4-methyl-2-chlorophenoxy)caproic acid is metabolized to (4-methyl-2-chlorophenoxy)butyric acid. Similarly, (2,4-dichlorophenoxy)caproic acid is metabolized to (2,4-dichlorophenoxy)butyric acid. Metabolism of 4-(4-chlorophenoxy)butyric acid to 3-hydroxy-4-(4-chlorophenoxy)butyric acid is followed by metabolic conversion to (4-chlorophenoxy)propionic acid. A similar pathway is followed by 4-(3-chlorophenoxy)butyric acid (462). These studies with strain T_{16} showed that 3-hydroxy acid intermediates appear during the metabolism of all the o-arylobutyric acids.

An alternative mechanism has been noted in *N. coeliaca* (432). Although β-oxidation is operative in this organism, α-oxidation operates in the metabolism of compounds with 10 or 11 side-chain carbons (phenoxydecanoic acid and phenoxyundecanoic acid). This process, demonstrated for nonchlorinated molecules, involves two enzymes: (a) a peroxidase catalyzing peroxidative decarboxylation of the fatty acid to yield CO_2 and the fatty aldehyde with one less carbon, and (b) a dehydrogenase catalyzing oxidation of the aldehyde to the corresponding acid.

The salt of phenoxy compound, sodium 2-(2,4-dichlorophenoxy)ethyl sulfate, is widely used in commercial formulations and is metabolized to 2-(2,4-dichlorophenoxy)ethanol by both *P. putida* FLA and cell-free filtrates of *Bacillus cereus* var. *mycoides* (279, 443). 2,4-D eventually appears in the *B. cereus* cell-free filtrates. Metabolism by *P. putida* does not result in production of 2,4-D. The enzyme of *P. putida* which breaks the oxygen-sulfur bond does not require prior activation (279). This is a novel alkylsulfatase, as other microbial alkylsulfatases break the chain at the carbon-oxygen bond.

FUNGAL METABOLISM OF PHENOXY HERBICIDES

Studies on fungal metabolism of phenoxy compounds have included studies with *Aspergillus niger*. Hydroxylation is a major mechanism, although not all vacant ring sites are hydroxylated. Thus, (2,4-dichloro-5-hydroxyphenoxy)acetic acid is the major metabolite of 2,4-D metabolism (Figure 51). A minor metabolite, (2,5-dichloro-4-hydroxyphenoxy)acetic acid, appears as the result of a novel hydroxyl-chloride replacement and chloride shift (148). The latter compound also is the only compound formed from metabolism of 2,5-D (148, 149). The hydroxyl-chloride shift is similar to that seen in 2,4-D metabolism by many plants, in which the major metabolite is (2,5-dichloro-4-hydroxyphenoxy)acetic acid and the minor metabolite is (2,3-dichloro-4-hydroxyphenoxy)acetic acid. Both metabolites require a hydroxyl-chloride shift (283).

Hendersonula toruloidea metabolizes 2,4-D with production of $^{14}CO_2$ (470). In 8 weeks, 28.8% of (carbon-1)-^{14}C 2,4-D and 2.8% of ring-^{14}C 2,4-D were released. *Stachybotrys atra* produced only 3% of (carbon-1)-^{14}C 2,4-D as $^{14}CO_2$ after 8 weeks.

Phytophthora megasperma var. *sojae* metabolized 10 mg/l 4-(2,4-D)B with 45% disappearance in 21 days, but no production of 2,4-D was noted (402). The organism also did not metabolize 2,4-D, suggesting that β-oxidation is not a primary mechanism in the metabolism of 4-(2,4-D)B.

MCPA is metabolized to (4-chloro-5-hydroxy-2-methylphenoxy)acetic acid by *A. niger* (149). The metabolism of 2- or 4-chlorophenoxyacetic acid by fungi does not result in ring cleavage, in contrast to the activity of bacteria (150). Metabolism of (4-chlorophenoxy)acetic acid yields compounds hydroxylated in the 2- or 3-

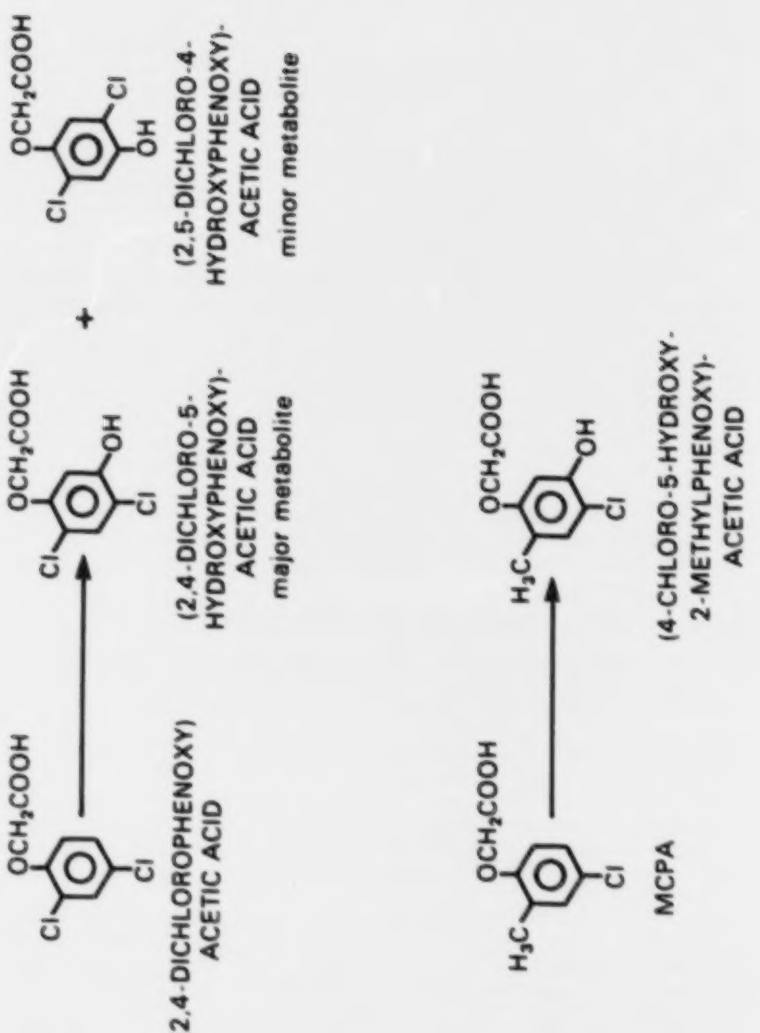

OCH$_2$COOH
Cl
Cl
(2,4-DICHLOROPHENOXY) ACETIC ACID
OCH$_2$COOH
Cl
Cl
OH
(2,4-DICHLORO-5-HYDROXYPHENOXY)-ACETIC ACID
major metabolite
+
OCH$_2$COOH
Cl
Cl
OH
(2,5-DICHLORO-4-HYDROXYPHENOXY)-ACETIC ACID
minor metabolite
OCH$_2$COOH
H$_3$C
Cl
MCPA
OCH$_2$COOH
H$_3$C
Cl
OH
(4-CHLORO-5-HYDROXY-2-METHYLPHENOXY)-ACETIC ACID

Figure 51 Metabolism of 2,4-D and MCPA by *A. niger*

positions, the latter a novel product. Similarly, (2-chlorophenoxy)acetic acid yields compounds hydroxylated in the 4- or 5-positions, the latter also a novel product. Minor products include 2-chloro-3-hydroxy and 2-chloro-6-hydroxy acids.

The microorganisms present in soil treated repeatedly with herbicides were isolated and identified (436). Bacteria capable of metabolizing 2,4-D include *Arthrobacter* sp., *Bacillus* sp., *Pseudomonas* sp., and *Sarcina* sp. Fungi include *Penicillium megasporum* and another *Penicillium* sp. Bacteria which can metabolize MCPA include *Arthrobacter* sp., *Corynebacterium* sp., and *Pseudomonas* sp., and fungi include *Fusarium culmorum*, *Mucor* sp., *Penicillium* sp., *Zygorhynchus moelleri* and four *Verticillium* spp. Of these, two bacteria and five fungi also metabolize 2,4-D.

METABOLISM OF PHENOXY HERBICIDES IN SOILS

A sample of Philippine soil was treated with 2,4,5-T for 4 months, after which 2,4,5-trichlorophenol was recovered (377). A mixture of microorganisms was removed and incubated with 2,4,5-T. Loss of 10% of substrate was recorded with liberation of 8% of the initial radioactivity of the uniformly ring-labeled substrate as $^{14}CO_2$ in 25 days. The major metabolite was 2,4,5-trichlorophenol, which was readily metabolized with about 75% of the chloride in this metabolite liberated as free chloride. About 40% of this compound was released as $^{14}CO_2$ in 25 days. Products arising from incubation of the mixed culture include 3,5-dichlorocatechol, *cis,cis*-2,4-dichloromuconic acid, 2-chloro-4-carboxymethylene but-2-enolide, chlorosuccinic acid, succinic acid, and 4-chlorocatechol. These products with the exception of 4-chlorocatechol, are all found in the 2,4-D degradative pathway subsequent to 3,5-dichlorocatechol (Figure 46).

The fate of uniformly ring-^{14}C-labeled 2,4-D and 2,4,5-T was explored in 6 different soils (304). Metabolites of 2,4,5-T included 2,4-5-trichlorophenol and 2,4-5-trichloroanisole, while no metabolites were detected after 2,4-D incubation. About 20 to 35% of the substrates were recovered from the humic and fulvic acids and humin fractions, indicating formation of polymeric humic substances of 2,4-D and 2,4,5-T mediated by additional hydroxyl groups on the rings. Depending on the soil, up to 83% of 2,4-D and 71% of 2,4,5-T applied at 1 ppm concentration was converted to $^{14}CO_2$ in 150 days.

Diclorfop-methyl, (±)-methyl 2-[4-(2,4-dichlorophenoxy)phenoxyl] propionic acid, undergoes rapid hydrolysis of the ester bond in field soils (297). At 1 ppm the resultant diclorfop is rapidly metabolized in aerobic soils with isolation of two metabolites when the ^{14}C-label is the chlorinated ring and one metabolite when the label is in the nonchlorinated ring. The ubiquitous metabolite was identified as 4-(2,4-dichlorophenoxy)phenol. Other experiments indicated that intermediates include dichlorfop acid with subsequent decarboxylation to form phenyl ether (397). In 25 weeks, 25 to 35% of each type of labeled substrate was converted to $^{14}CO_2$ (397). In anaerobic soils diclorfop persists with no evolution of CO_2 and formation of only trace amounts of a metabolite.

The herbicide 2-(2,4-dichlorophenoxy)ethanol is often applied to soils in the inert form sodium 2-(2,4-dichlorophenoxy)ethyl sulfate (443). In sterile soils conversion to the active form occurs only at pH 3 to 4, while in nonsterile soils conversion takes place at pH 3 to 7, within 45 minutes after application. Thus, in soils with pH greater than 4, herbicide activation is thought to be biologically mediated.

The isopropyl, n-butyl, and isooctyl esters of 2,4,5-T, the n-butyl ester of (2,4-dichlorophenoxy)butyric acid, and the isooctyl ester of (2,4-dichlorophenoxy)-propionic acid were applied to 4 Saskatchewan soils at 4 ppm concentration (400). In moist soils there was nearly complete conversion of the substrate to the free acids within 24 hours, with the exception of the isooctyl ester of (2,4-dichlorophenoxy)-butyric acid, which was completely converted in 72 hours. In air-dried soils there was very little loss of ester in the same time.

The isooctyl ester or dimethylamine salt of 2,4-D was applied to soils at a concentration of either 1.6 or 16 ppm (469). After 58 days 60 to 80% of the ring- or carboxyl-labeled substrate was released as $^{14}CO_2$, while 1% was recovered as 2,4-D. Eighty percent of the labeled substrate in the runoff water was recovered as $^{14}CO_2$ after 5 weeks, with an additional 3% more recovered in the next 5 weeks; the remaining material was not 2,4-D.

The primary effluent of municipal sewage was added to a nutrient medium containing 2,4-D (376). Within 7 days almost all the substrate disappeared. In a similar test phenoxyacetic acid disappeared within 12 days. Subsequent additions of either of these substrates resulted in metabolism without a lag period. No disappearance of 2,4,5-T was noted after 60 days.

Incubation of Maahas clay with medium containing these herbicides resulted in 90% disappearance of 2,4-D in 14 days on initial application, with 3 days required for 75% disappearance of additional applications of substrate (376). Phenoxyacetic acid required 16 days for initial disappearance, and subsequent applications were metabolized in 4 days. Evolution of $^{14}CO_2$ began from 7 to 60 days after herbicide application, and after 4 months from 5.2 to 34% was recovered depending on the soil.

After 12 weeks of incubation in sandy loam, 71 to 84% of either ring-^{14}C- or (carbon-1)-^{14}C- or (carbon-2)-^{14}C 2,4,-D was released as $^{14}CO_2$ (470). The concentration of substrate in the soil was not given.

Several South Vietnamese soil and mud samples were treated with carboxyl-^{14}C labeled 2,4,5-T (65). Two samples were thought to be treated previously with a 50:50 mixture of 2,4-D and 2,4,5-T, while two samples were thought to be uncontaminated. At 1 ppm concentration, almost 70% was evolved as $^{14}CO_2$ in 49 days. At 15 ppm, three soils converted 70 to 80% of the substrate to $^{14}CO_2$ in 168 days, while one sample, thought to be previously uncontaminated, evolved more than 95% of the material as $^{14}CO_2$.

In moist Philippine soils (upland conditions), 20 ppm 2,4-D disappeared more rapidly than when applied to flooded soils, but after 6 weeks the concentration of 2,4-D was similar in both moist and flooded soils (482). The same results were reported in the disappearance of 10 ppm 2,4,5-T from one of the soils. However, in another soil 2,4,5-T remained in flooded soils for a 4 week lag before undergoing rapid and complete disappearance in the next 4 weeks, while in the moist soils gradual disappearance was noted with about 40% remaining after 12 weeks. No disappearance of 2,4-D or 2,4,5-T was noted in sterile control samples after 12 weeks' incubation.

The persistence of 2,4-D and MCPA in soils following repeated applications was measured (436). Ten weeks were required for disappearance of 2,4-D upon first application. The herbicide disappeared after 7 weeks upon second application in the second year, and required only 4 weeks for disappearance after 18 years of repeated applications. MCPA required 20 weeks for disappearance in the first year, 10 weeks in the second year, and 7 weeks after 18 years. Soils pretreated with either herbicide showed accelerated 2,4-D disappearance after 18 years but not after one year of herbicide application. Enhancement of MCPA disappearance was noted after either one year or 18 years of pretreatment with either herbicide. The numbers of degradative bacteria or fungi were not significantly different after 0, 1 or 18 years of pretreatment.

A seed bioassay (mustard or cress) showed that 2,4-D disappears faster than MCPA, and 2,4,5-T much slower than either, from both a light clay soil and a sandy loam (414). The first application of 2,4-D to soil at 55 ppm concentration required 14 days for disappearance of herbicidal activity, while 7 days were required for disappearance of the second application at 120 ppm and 4 days upon third application of 200 ppm.

A soybean bioassay also indicated that 2,4,5-T lasted much longer than 2,4-D or MCPA (111). The rate of application had no effect at application rates from 5 to 20 lb/acre. The rate of disappearance of herbicide increased with increasing temperature and increasing moisture. Although MCPA is subject to degradation by photolysis, experiments with 1 mg/l MCPA incubated in rice water in the dark showed disappearance of 75% in 6 days, as opposed to 15% disappearance due to photolysis alone (405).

Application of bifenox, methyl 5-(2,4-dichlorophenoxy)-2-nitrobenzoate, to a greenhouse soil mix at a rate equivalent to 1.7 kg/ha showed that after 313 days 78% of the benzoate ring-^{14}C-labeled material and 67% of phenoxy ring-^{14}C-labeled material was bound to the soil (275). After 7 days following initial application very little additional bifenox disappeared, although only 20 to 26% was bound to the soils. The metabolites, which were identified by thin layer chromatography, included the acid of bifenox, 5-(2,4-dichlorophenoxy)-2-nitrobenzoic acid, nitrofen (2,4-dichlorophenoxy-4-nitrophenyl ether), 5-(2,4-dichlorophenoxy)anthranilic acid, and other unidentified compounds. These metabolites were also found as degradation products in plants grown in bifenox treated soil.

CHLOROPHENYL HERBICIDES

Pseudomonas sp. strain CBS 3 utilizes 4-chlorophenylacetic acid as the sole source of carbon and energy (258). Initially-formed metabolites include 4-chloro-3-hydroxyphenylacetic acid, 3-chloro-4-hydroxyphenylacetic acid and 4-chloro-2-hydroxyphenylacetic acid. This strain, however, cannot grow on 3-chloro-4-hydroxy or 4-chloro-3-hydroxyphenylacetic acid (296). Metabolism of 4-chloro-2-hydroxyphenylacetic acid results in formation of 4-chloro-2,3-dihydroxyphenylacetic acid. This is not the primary pathway of substrate metabolism. Upon further incubation 3,4-dihydroxyphenylacetic acid (homoprotocatechuate) appears, indicating direct removal and replacement of the chloride before ring cleavage. Homoprotocatechuate is metabolized to homogentisic acid (2,5-dihyroxyphenylacetic acid) and then to the *meta* cleavage product maleylacetoacetate resulting from the action of homogentisate 1,2-dioxygenase. An *Arthrobacter* sp. similarly produces 4-chloro-3-hydroxyphenylacetic acid from 4-chlorophenylacetate, along with an additional unidentified metabolite (110).

The herbicide chlorofenprop-methyl [2-chloro-3-(4-chlorophenyl) propionic acid methyl ester] is readily metabolized (264). In mixed cultures of soil microorganisms, 4-chlorocinnamic acid and 4-chlorobenzoic acid have been identified as metabolites. The latter product has been recovered from soil amended with the herbicide. Two strains, thought to be a *Flavobacterium* sp. and a *Brevibacterium* sp., were isolated from the soil and found to convert 4-chlorocinnamic acid to 4-chlorobenzoic acid. Two *Arthrobacter* spp. have been shown to grow on 4-chlorobenzoic as the sole source of carbon and energy. Thus, a consortia or a mixed soil population would be able to mineralize chlorofenprop.

A chlorophenyl insecticide known as SD 8280 [2-chloro-1-(2,4-dichlorophenyl)-vinyl dimethyl phosphate] was studied with respect to its degradation in soils (372). The major products were 2,4-dichlorobenzoic acid and 1-(2,4-dichlorophenyl)-ethanol. Lesser amounts of 2-chloro-1-(2,4-dichlorophenyl)vinyl methyl hydrogen phosphate and 2',4'-dichloroacetophenone were also formed. Other products were noted but not identified, although they were shown not to be 2,4-dichlorophenol or

2,4-dichlorobenzyl alcohol. None of the recovered metabolites represent alterations to the chlorinated ring of the molecule.

SUMMARY

Most of the chlorophenoxy herbicides appear to be biodegradable to CO_2 and free chloride under the right conditions. These results have been shown for both pure cultures and in soils containing mixed populations. Adaptation of the cultures to the substrate is required and results in faster disappearance of the compound. The persistence of some compounds in some environments indicates that under some conditions these herbicides could be considered to be recalcitrant compounds. Seasonal variations in herbicide degradation have also been noted (460).

Once a population has become adapted to metabolize a substrate, however, that capability persists for long periods of time. Thus, yearly applications of an herbicide are sufficient to maintain a degradative population in soil. A population adapted to metabolize a particular substrate is often also adapted to metabolize other related compounds. This has particular application where crop rotation is accompanied by usage of different herbicides.

SECTION 12

PHENYLAMIDE AND MISCELLANEOUS HERBICIDES

The phenylamide herbicides include the groups of phenyl ureas, N-phenylcarbamates, and acylanilides. Each takes the general form R-NH-CO-X where R is a halogenated or nonhalogenated aromatic hydrocarbon (239, 174). In urea herbicides, X is an amino group with methyl, alkyl, or methoxy substituents. Carbamates have the form whereby X is an alkoxy group. In acylanilide herbicides, X is an alkyl group. In many cases these herbicides are degraded to substituted anilines (305).

The urea herbicides are specific inhibitors of photosynthesis, but can have a selective effect because of their low water solubility and low mobility in soil. They can be applied to kill shallow-rooted weeds while having no effect on deeper-rooted plants of interest. In the late 1940s, a large number of substituted urea compounds were compared with 2,4-D for herbicidal activity and were found worthy of further development. Originally used as industrial weed killers, they have more recently been used in agricultural applications (174). The carbamates comprise a wide range of active compounds which, depending on the chemical substituents, are used for such purposes as herbicides, insecticides, and medicinals (186a). As insecticides, the carbamates inhibit the action of acetylcholinesterase, an enzyme required for proper neurotransmitter substance functioning. The degree of fit between the inhibitor and the enzyme lends selectivity to the activity of the carbamates. The herbicidal carbamates have also been used since the 1940s. Amide herbicides (substituted anilides) were developed in the 1950s and are used for such crops as corn and rice.

BACTERIAL METABOLISM OF CHLORINATED ANILINES

The degradation of such herbicides as monuron, diuron, linuron, and propanil results in formation of 3,4-dichloroaniline. A strain of *P. putida* has been isolated which mineralizes 3,4-dichloroaniline in the presence of aniline (483). The rate of mineralization was enhanced by increasing the concentration of aniline. In the presence of 500 mg/l propionanilide, as much as 50% of ring labeled chloroaniline (added at 10 to 60 mg/l concentration) was metabolized to $^{14}CO_2$ within 2 weeks, accompanied by some chloride release (484). A pathway for 3,4-dichloroaniline was proposed which involves *ortho* cleavage through 4,5-dichlorocatechol and further metabolism to succinic acid (Figure 52). This pathway is analogous to that shown for aniline. Dichloroaniline is also metabolized by a different mechanism to dichloroformylanilide (245, 484).

Another *Pseudomonas* sp., strain G, mineralizes 3,4-dichloroaniline to CO_2 when grown in the presence of 4-chloroaniline (488). In 9 days, 15% of 0.5 mM dichloroaniline was converted to CO_2.

Studies with 4-chloroaniline have shown that this metabolite is utilized as a sole source of carbon and nitrogen by *Pseudomonas* sp. strain G (487). After 10 days, 64% of a 2.5 ppm solution of the substrate was released as $^{14}CO_2$. Ammonium cation, rather than nitrate or nitrite anions, accumulates suggesting that the amino group is removed directly without oxidation. Other ^{14}C-labeled products accumulate but are

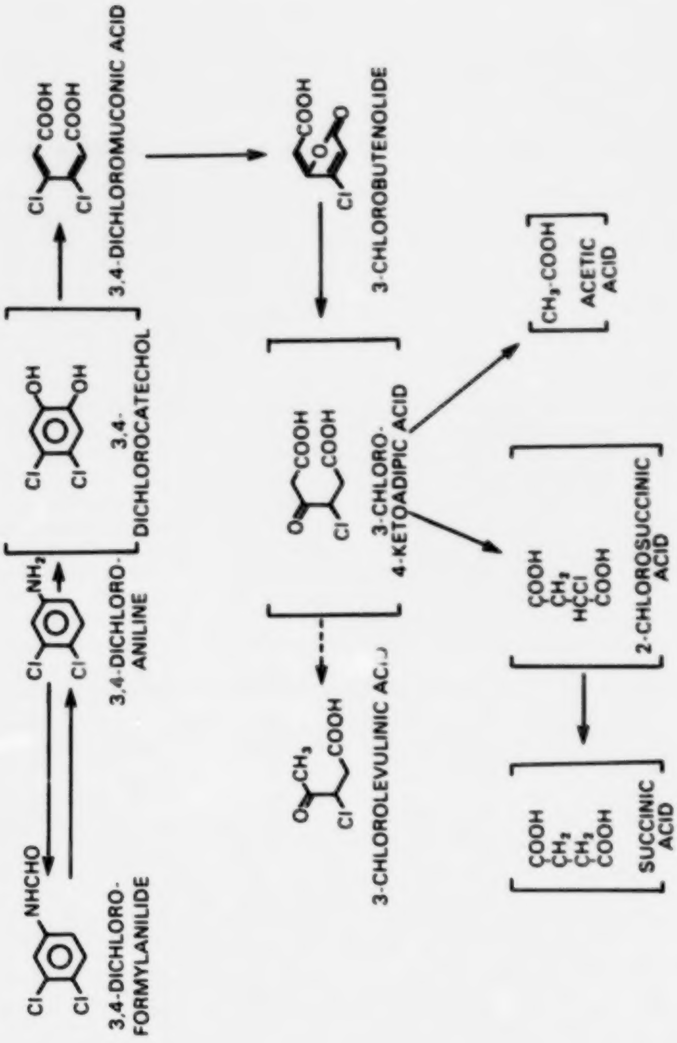

3,4-DICHLOROFORMYLANILIDE
NHCHO
Cl
3,4-DICHLOROANILINE
NH_2
3,4-DICHLOROCATECHOL
OH
3,4-DICHLOROMUCONIC ACID
COOH
3-CHLOROBUTENOLIDE
O
3-CHLORO-4-KETOADIPIC ACID
3-CHLOROLEVULINIC ACID
CH_3
2-CHLOROSUCCINIC ACID
HCCl
CH_2
SUCCINIC ACID
CH_3-COOH
ACETIC ACID

Figure 52. Pathways of 3,4-dichloroaniline metabolism by *P. putida*.

Adapted from Reference 484.

not incorporated into the cell biomass. Resting cells of this strain grown on 4-chloroaniline also oxidize aniline, catechol, and 4-chlorocatechol but not 4-chloronitrobenzene or 4-chlorophenol, indicating dioxygenase attack on the molecule (487). The *meta*-cleavage metabolite 2-hydroxy-5-chloromuconic semialdehyde also accumulates in the medium. This strain also utilizes 2-chloroaniline and 3-chloroaniline as sole sources of carbon and nitrogen. *P. multivorans* strain An 1, when grown with aniline present in the medium, converts 2-chloroaniline to 3-chlorocatechol, and 3-chloroaniline and 4-chloroaniline both to 4-chlorocatechol, which is metabolized subsequently to CO_2 and cell constituents (361).

A strain of *Alcaligenes faecalis* utilizes 3-chloroaniline or 4-chloroaniline under cooxidative conditions with sodium acetate or sodium pyruvate (419). Both chloroanilines are oxidized to 4-chlorocatechol, which undergoes *meta* cleavage to 5-chloro-2-hydroxymuconic semialdehyde and then to 2-chloro-4-oxalocrotonic acid. Similarly, an unidentified isolate which utilizes 4-chloro-aniline as the sole carbon source demonstrated, via Warburg respirometry, oxidation of 4-chlorocatechol without a lag (56).

The metabolism of 4-chloroaniline by *Paracoccus* sp. under both aerobic and anaerobic conditions was investigated (43). Transformation was faster under anaerobic conditions with 100% of a 20 mg/l solution converted within 2 days to a volatile product plus several other metabolites. The volatile product was not identified but was shown not to be CO_2. In the same period, 75% of the substrate was utilized aerobically, although the aerobic population was larger than the anaerobic population. The major product of aerobic metabolism is 4-chloroacetanilide, although several other products are formed as well. *Paracoccus* sp. also transforms 2-, 3-, 4-chloroaniline, 2,3-, 2,4-, 2,5-, and 3,4-dichloroaniline both aerobically and anaerobically, with more complete primary degradation occurring under anaerobic conditions.

A study of the anaerobic conversion of 4-chloroaniline by the same organism showed pH-dependent formation of products (310). In a medium containing nitrate, 80% of a 100 ppm solution was transformed within 48 hours to the condensation product 1,3-bis(p-chlorophenyl)triazine. However, sterile anaerobic solutions at pH of 5 to 6 also yielded this product, although no transformation took place at pH 7. It was postulated that the conversion of nitrate to nitrite and decrease in pH were the primary effects of bacterial metabolism, while triazine formation was a nonbiological secondary effect. *Paracoccus* sp. also formed small amounts of 4-chloroacetanilide during incubation with 4-chloroaniline.

A wide variety of both Gram-positive and Gram-negative bacteria were isolated from soil which had been enriched with 4-chloroaniline (134). The most active species was identified as *Bacillus firmus*. This species could not use the substrate as the sole source of carbon, but when grown with ethanol transformed 4-chloroaniline to 4-chloroacetanilide as the main metabolite, with lesser amounts of 4-chloropropionanilide also produced. Two other products were identified as 7-chloro-2-amino-3H-phenoxyazine-3-one and 7-chloro-2-amino-3H-3-hydroxyphenoxyazine, and were postulated to result from spontaneous condensation of 4-chloroaniline with subsequent hydroxylation. The acylation of the substrate described here is considered to be a detoxification process in microorganisms (134).

Aniline-grown resting cells of *Rhodococcus* sp. An 117 convert 2-chloro- and 3-chloroaniline to 3-chloro- and 4-chlorocatechol, respectively. An additional product identified as 2-chloromuconic acid results from metabolism of 2-chloroaniline. Cometabolism of 3-chloroaniline in the presence of 18O2 resulted in production of another product which was identified as the g-lactone of 3-hydroxymuconic acid, formed by incorporation of two molecules of oxygen (223). Appearance of this product was associated with disappearance of 4-chloro-catechol.

This suggests that dechlorination is associated with lactonization of the 3-chloromuconic acid, a mechanism shown previously in pseudomonads. When fresh cultures were supplied with benzoate plus 2- or 3-chloroaniline as the sole source of nitrogen, growth of cells and disappearance of both substrates occurred (222). The growth yield was similar to that obtained when NH_4NO_3 was utilized as the sole nitrogen source. No growth occurred when 4-chloroaniline was supplied as the nitrogen source.

The fungicide 2,6-dichloro-4-nitroaniline is metabolized by many bacteria including *E. coli* and *P. cepacia* (442). The first step is reduction of the nitro group to an amine, forming 2,6-dichloro-p-phenylenediamine. This compound is then acetylated to produce 4-amino-3,5-dichloroacetanilide. The first reductive step occurs much faster under anaerobic conditions than under aerobic conditions.

FUNGAL METABOLISM OF CHLORINATED ANILINES

Fungi as well as bacteria produce peroxidases which are responsible for the polymerization of chloroanilines. Species with this capability include *Geotrichum candidum* L-3 and *Aspergillus* sp. (47, 271). The substrate 3,4-dichloroaniline is converted to 3,3′,4,4′-tetrachloroazobenzene by *G. candidum* L-3 and *Aspergillus* sp. (271) and is converted to 3,3′,4,4′-tetrachloro-azoxybenzene in *Fusarium oxysporum* cultures (243). However, this latter reaction varies with the culture conditions and the azoxy condensation product has not been found in soils.

The fungal metabolism of 4-chloroaniline follows several pathways (Figure 53). A major pathway is N-hydroxylation such as is demonstrated by *F. oxysporum* (242). This species metabolizes 4-chloroaniline to 4-chlorophenylhydroxylamine which is subsequently converted to 4-chloronitrosobenzene and 4-chloronitro-benzene. Condensation products which appear include both 4,4′-dichloroazo-benzene and 4,4′-dichloroazoxybenzene. In addition, 4-chloroacetanilide appears as an acylation reaction and may undergo hydrolysis to yield 4-chloroaniline. Free chloride is produced as the result of some of these reactions.

The culture filtrate of *G. candidum*, as well as the purified fungal enzymes peroxidase and aniline oxidase, converts 4-chloroaniline to several condensation products including 4,4′-dichloroazobenzene and 4-chloro-4′-(4-chloroanilino)-axobenzene (49). These reactions have also been demonstrated with horseradish peroxidase. *Streptomyces* sp. also formylates 4-chloroaniline with resultant production of 4-chloroformylanilide as well as 4-chloroacetanilide and at least two other metabolites (381).

Ring hydroxylation is a mechanism demonstrated by *F. oxysporum* which results in metabolism of 3-chloroaniline to 2-amino-4-chlorophenol and 4-chloroaniline to 2-amino-5-chlorophenol (Figure 53) (155). These molecules are hydroxylated in the *ortho* position. The aminophenols are relatively unstable and can undergo condensation and polymerization reactions, although 2-amino-4-chlorophenol has been detected in soil as well as in the pure culture studies reported above. The *ortho*-substituted substrate 2-chloroaniline is not hydroxylated by *F. oxysporum*.

METABOLISM OF CHLORINATED ANILINES IN SOILS

There is evidence that in soils 3,4-dichloroaniline is slowly mineralized. Radiocarbon-labeled humic-bound material is mineralized in some soils ($^{14}CO_2$ production) at about the same rate as the average soil organic matter polymer (384). The addition of aniline to soils enhances mineralization of both free and humic-bound 3,4-dichloroaniline (483). This was attributed to selection by the aniline analogue for chloroaniline-degradative populations as well as the induction by aniline of the common metabolic pathway.

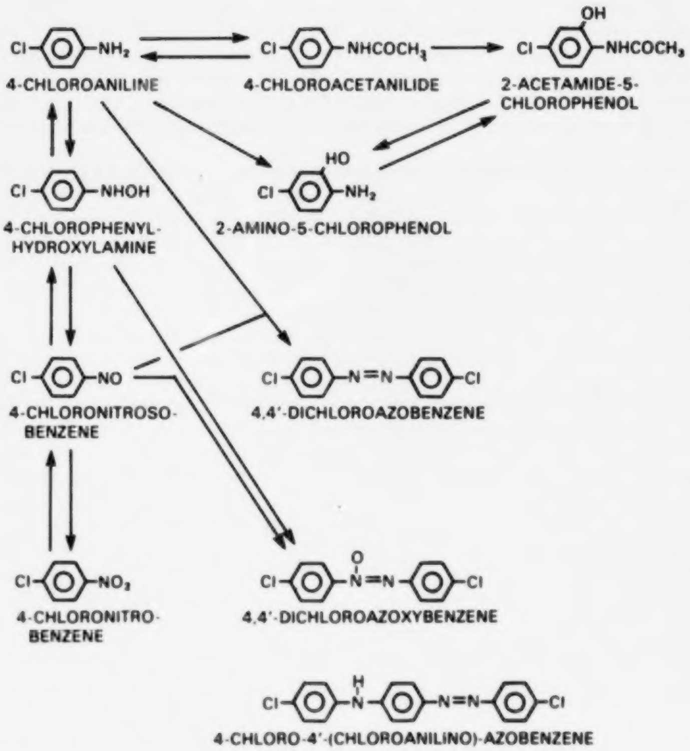

Cl NH₂
4-CHLOROANILINE
Cl NHCOCH₃
4-CHLOROACETANILIDE
OH
Cl NHCOCH₃
2-ACETAMIDE-5-CHLOROPHENOL
Cl NHOH
4-CHLOROPHENYL-HYDROXYLAMINE
HO
Cl NH₂
2-AMINO-5-CHLOROPHENOL
Cl NO
4-CHLORONITROSO-BENZENE
Cl N=N Cl
4,4'-DICHLOROAZOBENZENE
Cl NO₂
4-CHLORONITRO-BENZENE
O
Cl N=N Cl
4,4'-DICHLOROAZOXYBENZENE
H
Cl N N=N Cl
4-CHLORO-4'-(CHLOROANILINO)-AZOBENZENE

Figure 53. Metabolism of 4-chloroaniline by microorganisms.

Adapted from References 49, 155, 242.

Application of 10 ppm ring-labeled ^{14}C-3,4-dichloroaniline to a rice paddy ecosystem yielded a total recovery (extractable plus nonextractable) of almost 69% (219). Less than 4% of the material applied to soil was recovered in the water or rice plants. CO_2 evolution was not determined. Extraction of the soil yielded 3.4% of the original material as dichloroaniline, 4% as tetrachloro-azobenzene, and 44.7% as polar material.

The degradation of aniline, 4-chloroaniline, and 3,4-dichloroaniline in four different soils was compared (420). At 1 ppm application rate, 16 to 26% aniline was converted to CO_2 after 10 weeks, while after 16 weeks 12 to 27% chloroaniline and 4 to 12% dichloroaniline were mineralized. In soils 4-chloroaniline can be converted to 4-chlorophenylhydroxylamine via biological mechanisms (48). This compound then undergoes condensation with 4-chloroaniline to form 4,4′-dichloroazobenzene in a nonbiological reaction.

Condensation of two molecules of 3,4-dichloroaniline forms 3,3′,4,4′-tetrachloroazobenzene which is relatively persistent in the environment (51). This reaction has been demonstrated in Nixon sandy loam (11, 23, 24) as well as in other soils (22, 48, 51, 245). The conversion of chloroanilines to chloroazobenzenes has been shown to occur by a peroxidase mechanism (25). A mixture of substituted anilines, hydrogen peroxide, and peroxidase resulted in the formation of chloroazobenzene (51). Bacteria including *Bacillus* sp., *Arthrobacter* sp., and *Pseudomonas* sp. exhibit peroxidase activity (271). A pathway has been proposed which includes transformation of the hypothetical 3,4-dichloroanilidyl molecules to 3,4-dichlorophenylhydroxylamine. Two of these molecules are condensed to the hypothetical 3,3′,4,4′-tetrachlorohydrazobenzene which is converted to 3,3′,4-4′-tetrachloroazobenzene (51). However, studies in which dichloroaniline was applied to herbicide-treated soils did not show formation of tetrachloroazobenzene, suggesting that dichloroaniline is not the prime precursor for tetrachloroazobenzene (30). Also, production of tetrachloroazobenzene in soils incubated with propanil was not correlated with the quantity of peroxidase-producing microorganisms recovered from the soil (58). Cell-free perioxidase was found only rarely in the soil samples. Recovery of peroxidase increased upon amendment of the soils with nutrient sources and additionally upon sonification of the samples, which may have released cell-bound or intracellular enzymes. Addition of proteose-peptone decreased recovery of peroxidase-producing organisms but increased soil peroxidase recovery. However, this peroxidase did not form tetrachloroazobenzene from dichloroaniline. Another condensation product detected as a humic-bound residue in soils treated with propanil is 4-(3,4-dichloroanilino)-3,3′,4′-trichloroazobenzene (22).

Warburg respirometry studies indicated that aniline-acclimated activated sludge microflora oxidized 500 mg/l 2-chloroaniline at low rates over a 192-hour incubation period (293). Oxidation of 4-chloroaniline occurred at slightly higher rates, and after a 100-hour lag period rapid oxidation of 3-chloroaniline took place.

METABOLISM OF UREA HERBICIDES

A *Fusarium* sp. utilizes the larvacide diflubenzuron [1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea] as its sole source of carbon and energy (389). The pathway was elucidated to include initial formation of 2,6-difluorobenzoic acid and 4-chlorophenylurea. The latter compound is metabolized to 4-chloroaniline, and then 4-chloroacetanilide, followed by reductive dehalogenation to acetanilide and further metabolism to cell constituents. Other fungi including *Cephalospocium* sp., *Penicillium* sp., and *Rhodotorula* sp., although unable to utilize diflubenzuron as a sole carbon source, metabolized the compound to 2,6-difluorobenzoic acid and 4-chlorophenylurea, indicating cleavage of the urea bridge.

Evidence that the urea herbicides are degraded in the environment accrues from studies which showed that such compounds as 3-(4-chlorophenyl)-1,1-dimethylurea, (monuron) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron), when applied at rates of 1 to 2 lb/acre annually in the eastern part of the United States, left no residual phytotoxicity after 4 to 8 months (203). Higher application rates required longer times for disappearance of phytotoxicity.

Potential mechanisms for disappearance include biological, leaching, volatilization and chemical decomposition (203). Leaching is considered to be significant only in porous soil or if there is a great amount of rainfall. The low vapor pressure and aqueous solubilities of these herbicides make volatilization unlikely to be an important mechanism. Photodecomposition may be a factor in dry areas in which the herbicide remains on the soil surface, but these compounds are stable to chemical decomposition in aqueous solutions. Biological studies revealed that the rate of herbicide inactivation is greater in nonsterile than in sterile soils. This was shown by the amount of radiocarbon-labeled CO_2 evolved from soils amended with ^{14}C(methyl)-labeled monuron, and a *Pseudomonas* sp. was isolated which oxidized this substrate in Warburg respirometry studies (203).

The herbicide N′-(4-chlorophenoxy)phenyl-N,N-dimethylurea is metabolized when placed in contact with soils such as sandy loam or humus soil (173). Sorption of the compound to the soils has an effect on the rate of degradation. Enriched bacterial cultures derived from the soil samples also metabolized the herbicide by successive demethylation to N′-(4-chlorophenoxy)phenyl-N-methylurea and subsequently to N′-(4-chlorophenoxy)phenylurea. No appreciable amounts of CO_2 were released. Fungal isolates of *Penicillium* sp. and *Aspergillus* sp. removed less than 50% of the carbonyl group but did not transform the compound further. The metabolism of this herbicide in plants also follows successive demethylation leading to CO_2 evolution.

Diuron is used for long-term weed control in peach orchards, and has been detected as long as 3 years after the last application in a field consisting of Fox loamy sand (248). The levels of 3,4-dichloroaniline were very low and decreased to undetectable levels in 3 years, while the potential condensation product tetrachloroazobenzene was not detected. The decomposition of diuron is enhanced by changes in environmental conditions that favor the growth of microorganisms (306). Thus, increasing the temperature of incubation from 10 to 30°C or adding organic matter to the soils both increase the rate of diuron decomposition. The rate of herbicide inactivation is much greater than the rate of CO_2 evolution, and investigations showed that the loss of one methyl group decreases herbicidal activity by half while loss of both methyl groups completely inactivates the molecule. The pathway of diuron metabolism in soils was proposed to be 3-(3,4-dichlorophenyl)-1,1-dimethylurea to 3-(3,4-dichlorophenyl)-1-methylurea, then loss of the second methyl group to form 3-(3,4-di-chlorophenyl)urea, followed by hydrolysis of the urea to form the aniline derivative 3,4-dichloroaniline, which accumulates as the major product (103).

Microbial enrichment cultures from pond water and pond sediment treated with diuron revealed a similar pathway of degradation (131, 132). Three additional unidentified products were also detected. The enrichment cultures included mixtures of fungi and bacteria as well as consortia of bacteria. Some of the mixed cultures converted more than 90% of the substrate to CO_2 within 3 weeks. Of 20 single isolates, however, only 3 could partially metabolize diuron after 4 weeks' incubation.

Under anaerobic conditions reductive ring dechlorination of diuron occurs (12). Enrichment cultures from pond water and sediment incubated anaerobically rapidly degraded diuron to 3-(3-chlorophenyl)-1,1-dimethylurea in stoichiometric amounts. This product was not degraded further and no other products were detected. Repeated additions of diuron resulted in rapid metabolism of the substrate.

Chlortoluron [N-(3-chloro-4-methylphenyl)-N'-dimethylurea] has a half-life in a variety of soils of 4 to 6 weeks (403). The major degradation product is monomethyl chlortoluron. However, the product of subsequent demethylation was never detected. This may indicate that cleavage of the molecule to form 3-chloro-4-methylaniline follows monomethyl chlortoluron formation. Although the substituted aniline was not detected, this product rapidly disappears from the soils when applied directly.

Methoxy phenyl urea herbicides have quite high herbicidal selectivity and additionally are not very persistent in soil after application (174). Metabolism of these compounds differs from that of the dimethyl phenyl urea herbicides.

Soils treated with 3-(4-chlorophenyl)-1-methoxy-1-methylurea (monolinuron) yielded a *Bacillus sphaericus* isolate which could cometabolize ^{14}C(ureido)-monolinuron to $^{14}CO_2$ and 4-chloroaniline (452). Maximum degradation occurred after the end of logarithmic growth. The substituted aniline was not degraded by the organism but was lost from the culture through volatilization. Linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea] was metabolized to stoichiometric amounts of 3,4-dichloroaniline, while the dimethyl compounds monuron and diuron were not degraded. Cell-free extracts of *B. sphaericus* also transformed the methoxy herbicides to substituted anilines with the aliphatic portion of the molecule degraded to CO_2 plus another metabolite (453). The degradation product of linuron was identified as N,O-dimethylhydroxylamine (133). The cell-free extracts were less active against the dimethyl substrates monuron and diuron (453).

The soil fungus *Cunninghamella echinulata* Thaxter degrades linuron and monolinuron through stable hydroxymethyl intermediates (435). Linuron is metabolized to 3-(3,4-dichlorophenyl)-1-methoxy-1-hydroxymethylurea and subsequently 3-(3,4-dichlorophenyl)-1-methoxyurea and 3-(3,4-dichlorophenyl)-1-methylurea. Disappearance of compounds such as linuron from nonsterile, but not from sterile, soils has been noted (120). The degradation of monolinuron during waste composting was investigated (318). After three weeks of composting, N-methoxy-N'-4-chlorophenylurea was present in trace amounts and was the only metabolite detected.

METABOLISM OF CHLORINATED PHENYL CARBAMATE HERBICIDES

The phenyl carbamate herbicides (also known as carbanilates) are used to kill weeds on crop plants such as rice. Substituted anilines arise from degradation of these compounds, as the primary mechanism of degradation seems to be hydrolysis of the ester linkage (239).

The cell-free enzyme extract from a *Pseudomonas* sp. isolated from a soil enrichment culture was capable of converting several chlorophenylcarbamates to corresponding chlorinated anilines (244). The compounds tested included CIPC, [isopropyl-N-(3,4-dichlorophenyl)carbamate],sec-butyl-N-(3,4-dichlorophenyl)-carbamate, α-carboisopropoxyethyl-N-(3-chlorophenyl)carbamate, 2-chloroethyl-N-(3-chlorophenyl)carbamate, 2-(1-chloropropyl)-N-(3-chlorophenyl)carbamate, 2-ethylhexyl-N-(3-chlorophenyl)carbamate and α-carbo-(2,4-dichlorophenoxyethoxy)-ethyl-N-(3-chlorophenyl)carbamate. In contrast, the enzyme preparation had no activity against 3-(4-chlorophenyl)-1,1-dimethylurea (monuron).

Penicillium jenseni was isolated from soil which had been treated with barban [(3-chlorophenyl)carbamic acid 4-chloro-2-butyl ester] (472). The mold did not utilize barban as a carbon source, although trace amounts of 3-chloroaniline appeared after incubation. Incubation of the mycelia with barban resulted in production of large amounts of 3-chloroaniline, which was metabolized by the mycelia suspensions without production of free chloride.

A mixture of bacteria and fungi isolated from soil enriched with the nonchlorinated analogue isopropyl phenylcarbamate (propham) metabolized a wide range of herbicides to their corresponding chlorinated aniline products (305). These included compounds ring-substituted with 3-chloro-, 4-chloro-, 2,4-dichloro-, 3,4-dichloro-, and 2,4,5-trichloro- substituents. Microorganisms comprising the consortium included *Mycobacterium* sp., *Arthrobacter* sp., *Corynebacterium* sp., *Fusarium* sp., *Nocardia* sp., *Streptomyces* sp., *Aspergillus* sp., and *Penicillium* sp.

A wide variety of fungal isolates from treated soil metabolized swep [methyl N-(3,4-dichlorophenyl)carbamate] to 3,4-dichloroaniline with formation of trace amounts of 3,3′,4,4′-tetrachloroazobenzene (240). The fungi included *Aspergillus ustus, A. versicolor, Fusarium oxysporum, F. solani, Penicillium chrisogenum, P. nigulosum* and *Trichoderma viride*. The isolates were most active on CIPC and only slightly active against the phenylureas diuron and 3-(3,4-dichlorophenyl)-1-methylurea. The rate of formation of 3-chloroaniline was the same as the rate of disappearance of CIPC due to metabolism by *Aspergillus fumigatus*, indicating that hydrolysis of the ester bond is the first step in degradation of this herbicide (471).

Swep is metabolized to 3,4-dichloroaniline with formation of 3,3′,4,4′-tetrachloroazobenzene in soils such as Nixon sandy loam (11). Soil microorganisms obtained from muck soil metabolized 1240 mg/l isopropyl-N-(3-chloro-phenyl)carbamate (CIPC, chlorpropham) to 3-chloroaniline with complete chloride release within 13 days (241). A similar pathway with complete dechlorination within 16 days was observed in the metabolism of 2-chloroethyl-N-(3-chloro-phenyl)carbamate (CEPC). The isolates lost degradative capability if they were maintained on nutrient agar for several days but they could be readapted to use these herbicides as a sole source of carbon.

METABOLISM OF ACYL ANILIDE HERBICIDES

The substituted anilide herbicides are structurally related to the urea herbicides and the carbanilates, and like them are degraded to substituted anilines.

Strains of *Pseudomonas striata* and *Achromobacter* sp. metabolize N-(3-chloro-4-methylphenyl)-2-methylpentanamide(3′-chloro-2-methyl-*p*-valero-toluidide, solan) to 3-chloro-*p*-toluidine from which chloride is released quantitatively (Figure 54) (240). Azobenzene products were not detected in these experiments.

Fungi from several genera, including *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp., and *Trichoderma* sp. also metabolize solan to 3-chloro-*p*-toluidine with chloride release (240). Cultures of *A. niger* metabolize solan to a product identified as 3′-chloro-4′-methylacetanilide (Figure 45) (455). Cell-free extracts converted solan to the substituted aniline, but in growing cultures this product was rapidly acetylated to effect detoxification and the free aniline could not be recovered.

Strains of *P. striata* and *Achromobacter* sp. metabolize both swep and N-(3,4-dichlorophenyl)propionamide (propanil) to 3,4-dichloroaniline and small quantities of 3,3′,4,4′-tetrachloroazobenzene (240). *Corynebacterium pseudodiphtheriticum* NCIB 10803 utilizes propanil as the sole source of carbon and energy for growth (186). The resulting products are 3,4-dichloroaniline which accumulates in the medium, and the propionic acid moiety which is utilized for cell growth.

A strain of *F. solani* was isolated which utilizes propanil as a sole source of carbon and energy (269). Dichloroaniline accumulates in the medium until it reaches toxic levels. The enzyme responsible for propanil hydrolysis to propionate and dichloroaniline was identified as an acylamidase (270). This enzyme is specific for molecules with a short chain, as it could not hydrolyze dicryl [N-(3,4-dichlorophenyl)metha-

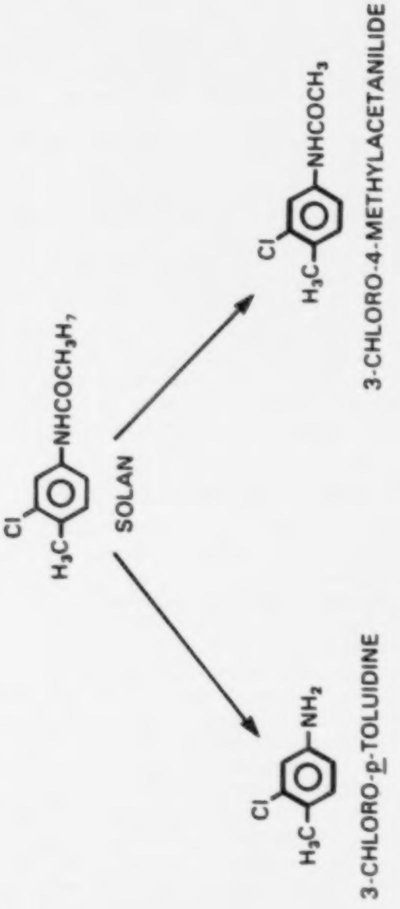

SOLAN
3-CHLORO-4-METHYLACETANILIDE
3-CHLORO-p-TOLUIDINE

Figure 54. Metabolism of solan by microorganisms.

Adapted from References 240, 455.

crylamide] or the six-carbon herbicide 2-methylpentanamide. Other fungi, including *Aspergillus ustus, A. versicolor, Fusarium oxysporum, F. solani, Penicillium chrysogenum, P. nigulosum* and *Trichoderma viride*, also metabolize propanil to 3,4-dichloroaniline with formation of trace amounts of 3,3′,4,4′-tetrachloroazobenzene (240).

The interaction of *Penicillium piscarium* and *Geotrichum candidum* incubated with propanil results in increased growth over either alone (50). *P. piscarium* contains an acylamidase which converts propanil to dichloroaniline. *G. candidum* cannot utilize propanil but contains a peroxidase which converts dichloroaniline to the tetrachloroazobenzene. Each fungus reduces the toxic level of the other's byproduct of metabolism, demonstrating a synergistic or mutualistic interaction.

The yeast *Pullularia pullulans* and two *Penicillium* spp. were isolated and found to utilize N-(3,4-dichlorophenyl)-2-methylpentanamide as a sole source of carbon and energy, metabolizing the herbicide to dichloroaniline and 2-methyl-valeric acid (392). The enzyme was inducible and differed in level of activity and substrate specificity among the three species. Cell-free extracts of one of the *Penicillium* spp. hydrolyzed a wide variety of other acyl anilides as well, but had no activity against diuron or CIPC.

An unusual product, identified as N-(3,4-dichlorophenyl)-2-methyl-2,3-dihydroxypropionamide, was detected in the culture medium of a *Rhizopus japonicus* culture growing in the presence of dicryl (456). This product results from the double hydroxylation of the ethylene double bond of dicryl, and was the only metabolite detected. *R. japonicus* also hydroxylates the side chain of N-(3,4-dichlorophenyl)-pentanamide to produce N-(3,4-dichlorophenyl)-3-hydroxy-2-methylpentanamide (457). This mechanism results in detoxification of the herbicide.

Since propanil is an inhibitor of photosynthesis it is a potential poison to the algae as well (473). A study of the effect of propanil on cyanobacteria indicated depression of photosynthesis but also showed conversion of propanil to the less toxic 3,4-dichloroaniline in both axenic (bacteria-free) and contaminated cultures. The species studied incorporated those found in flooded rice paddy fields and soil, and included *Anabaena cylindrica, A. variabilis, Nostoc muscorum, N. entophytum, Tolypothrix tenuis*, and *Gloeocapsa alpicola*.

In soils propanil is converted to 3,4-dichloroaniline and the condensation product 3,3′,4,4′-tetrachloroazobenzene (30, 87). Uniformly-labeled ^{14}C-propanil applied to soils was transformed to 3,4-dichloroaniline and the multiple condensation product 4-(3,4-dichloroanilino)-3,3′,4′-trichloroazobenzene which accumulated to 2% of the substrate (22). At high concentration (500 mg/l), the dichloroaniline was volatile, while at lower concentrations (5 to 10 mg/l) dichloroaniline and its condensation product were humic-bound. The aliphatic portion is degraded to CO_2. Studies with ^{14}C(carbonyl)-propanil revealed 70% conversion to $^{14}CO_2$ within 25 days, while soils amended with ^{14}C(ring)-propanil yielded only 3% $^{14}CO_2$ during the same time period (87). *Pseudomonas* sp. strain G also converts propanil to 3,4-dichloroaniline (488).

Propanil sprayed onto flooded rice plots was dissipated quickly and disappeared within 24 hours (112). The major metabolite was 3,4-dichloroaniline which sorbed to soils. Only a trace of 3,3′,4,4′-tetrachloroazobenzene was detected, although dilution caused by the flooding may have precluded condensation of the dichloroaniline.

The amount of tetrachloroazobenzene formed in soils as a result of propanil or 3,4-dichloroaniline application was found to be highly variable (213). In a comparison of 9 soils, those at a pH of 4.5 to 5.5 showed the most production. Tetrachloroazobenzene formation was not correlated with the organic matter content of the soils, and air-dried soil samples showed 87 to 99% reduction in product formation. More tetrachlorozobenzene was produced from direct applicaton of dichloroaniline than from molar equivalent application of propanil. In a contradictory study, however, more tetrachloroazobenzene was produced from propanil than from the equivalent application of 3,4-dichloroaniline (58).

The degradation rates of herbicides in Nixon sandy loam have been correlated with the number of carbon atoms in the side chain (23). The four-carbon molecule dicryl is metabolized at a slower rate than propanil, and the six-carbon herbicide N-(3,4-dichlorophenyl)-2-methylpentanamide is the most persistent of the three. Each is converted to 3,4-dichloroaniline, 3,3′,4,4′-tetrachloroazobenzene and another metabolite.

MISCELLANEOUS PESTICIDES

Chlordimeform [N′-(4-chloro-e-methylphenyl)-N,N-dimethylmethanimideamide] represents another class of formamide compounds used as insecticides. Chlordimeform is metabolized in sandy loam to several products within 90 days (220). These include 4-chloro-6-nitro-*o*-toluidine and 4-chloro-*o*-toluidine (Figure 46). The latter product is condensed to form 4,4′-dichloro-2,2′-dimethylazobenzene. Other coupling products which were detected include N-(4-chloro-*o*-tolyl)-2-methyl-*p*-benzoquinone monoimine and 2-(4-chloro-*o*-toluidino)-N-(4-chloro-*o*-tolyl)-6-methyl-*p*-benzoquinone monoimine. These are formed by a one-electron oxidation mediated by peroxidases, and appearance of these products is pH-dependent.

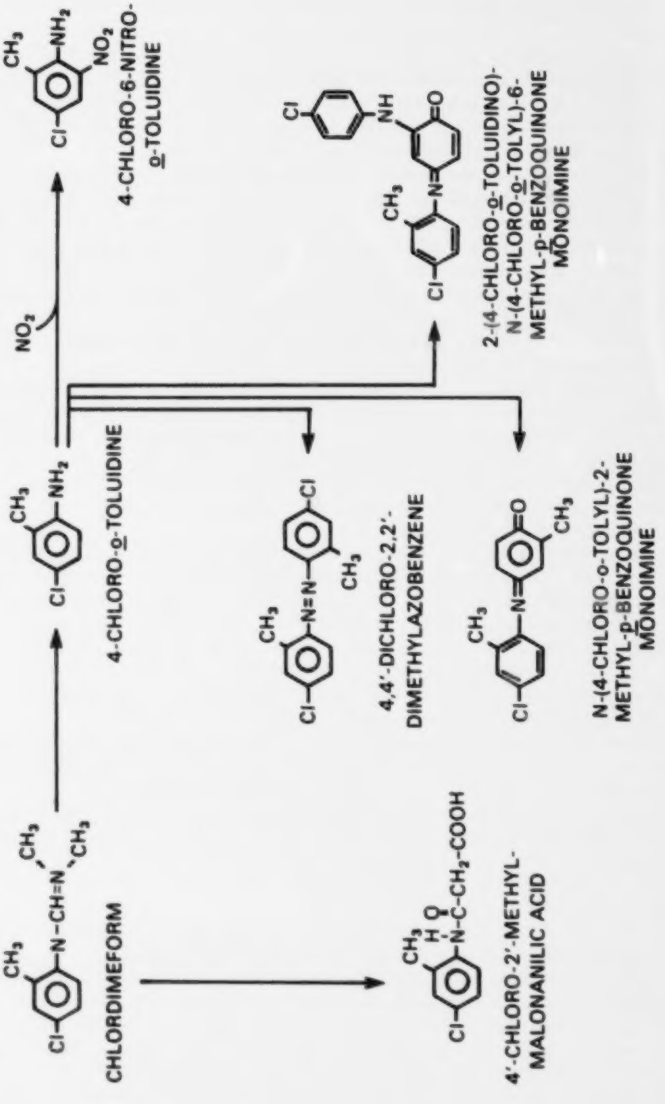
A mixed population of soil microorganisms mediated a novel conjugation of the aniline moiety of chlordimeform with malonic acid to form 4′-chloro-2′-methylmalonanilic acid (Figure 55) (378). This mechanism was previously found only in plants as a means for detoxification of certain D-amino acids.

The herbicide N-(1,1-dimethylpropynyl)-3,5-dichlorobenzamide underwent extensive metabolism of the side chain during 90 days' incubation in soils (481). However, no alteration of the chlorinated ring structure was noted. The cyanobacterium *Oscillatoria* sp. metabolized N'-(4-chloro-*o*-tolyl)-N,N-dimenthylformamidine with production of $^{14}CO_2$ from either tolyl-^{14}C- or ring-^{14}C-labeled substrate (34). Extensive nonbiological degradation of this compound occurs but no evolution of $^{14}CO_2$ in the absence of the algae was noted.

Techlofthalam [N-(2,3-dichlorophenyl)-3,4,5,6-tetrachlorophthalamic acid] is a bactericide used on rice plants (252). An analysis of its fate under flooded soil conditions analogous to those of rice paddy fields indicated that after 32 weeks most of the recoverable radiolabeled material was isolated as two or more products chlorinated in the tetrachlorophthalamic ring. Nine percent of the carboxyl-labeled material was converted to $^{14}CO_2$. Techlofthalam was recovered as a minor metabolite. No further transformations occurred during the 32 weeks of the experiments.

Metabolism of chlomethoxynil (2,4-dichlorophenyl-3′-methoxy-4′-nitrophenyl ether) in flooded soil resulted in production of a number of compounds due to alteration of the molecule without loss of chloride (328). Cleavage of the ether bond results in production of 2,4-dichlorophenol.

Production of amino derivatives from other diphenyl ether herbicides was shown to be faster in flooded than in moist soils (329). A number of bacteria including those from the genera *Bacillus*, *Pseudomonas*, *Enterobacter* and *Escherichia* mediated this conversion, although disappearance of the herbicides was noted in sterile soils as well. The herbicides tested included nitrofen (2,4-dichlorophenyl 4′-nitrophenyl ether), 2,4,6-trichlorophenyl 4′-nitrophenyl ether, 2,4-dichloro-6-fluorophenyl 4′-nitrophenyl ether, and chlomethoxynil.



CHLORDIMEFORM
4-CHLORO-o-TOLUIDINE
NO_2
4-CHLORO-6-NITRO-o-TOLUIDINE
4'-CHLORO-2'-METHYL-MALONANILIC ACID
4,4'-DICHLORO-2,2'-DIMETHYLAZOBENZENE
N-(4-CHLORO-o-TOLYL)-2-METHYL-p-BENZOQUINONE MONOIMINE
2-(4-CHLORO-o-TOLUIDINO)-N-(4-CHLORO-o-TOLYL)-6-METHYL-p-BENZOQUINONE MONOIMINE

Figure 55. Metabolism of chlordimeform in soils.

Adapted from References 220, 378.

SUMMARY

The herbicides described here were formulated with degree of persistence after application as a prime consideration. All of these compounds undergo primary degradation readily in soils and in a variety of microbial cultures. The resulting products are chlorinated anilines and other metabolites arising from the aliphatic side chain. When the side chain is not chlorinated, it is metabolized to cell constituents and CO_2. Little research has been published regarding the fate of chlorinated or other highly substituted side chain metabolites.

The bulk of the herbicides remain as chlorinated anilines. There is some evidence for volatilization of these compounds in arid zones if sorption to soils is delayed or if the concentration of chloroanilines is very high. However, the chlorinated anilines are readily and strongly bound to soil humic substances. Monosubstituted anilines, particularly 3-chloroaniline and 4-chloroaniline, are utilizable as sole carbon sources by some microorganisms and are metabolized in laboratory experiments. Dichloroaniline is toxic to microorganisms at low concentrations and evidence for its degradation is indirect. It is not clear how sorption to soils affects degradation of the chloroanilines in the environment.

Chlorinated anilines are metabolized by dioxygenases to chlorocatechols. Organisms which convert the chlorinated anilines to chlorocatechols and then also metabolize the chlorocatechols do so by the *meta* pathway. Limited evidence suggests that the amine group is removed directly without oxidation.

Under some conditions azobenzenes are formed from condensation of chloroanilines via peroxidases. The concentration of chloroanilines must be very high for this mechanism to be operative in soils.

Primary degradation of the urea herbicides occurs by successive demethylation of the side chain. The side chain is then metabolized leaving the chloroaniline moiety. Methoxy phenyl ureas are metabolized by a different mechanism, as microorganisms active against these compounds have little activity against the dimethyl herbicides.

Reductive dechlorination to a limited extent of the aromatic moiety has been reported. However, the effectiveness of this mechanism in the environment has not been investigated. Anaerobic utilization of 4-chloroaniline takes place readily, but the resultant volatile product or products have not been identified.

The primary mechanism of degradation of the phenyl carbamate herbicides is hydrolysis of the ester linkage to form chlorinated anilines. These compounds are metabolized by a wide variety of fungi and bacteria as noted above.

Hydrolysis of the acyl anilide herbicides results in formation of the chlorinated anilines with utilization of the aliphatic side chain for cell growth. An unusual mechanism employed by *R. japonicus* results in hydrolysis of the side chains of dicryl and N-(3,4-dichlorophenyl)-2-methyl pentanamide. This modification results in detoxification of the molecules.

Studies with various species of algae have shown that these organisms metabolize acyl anilide compounds with production of chlorinated anilines.

Metabolism of these classes of herbicides is dependent upon factors which affect microbial soil activity, including such factors as pH and soil composition which affect the chemical state of the compound as well. These compounds are relatively easily broken down to chlorinated anilines and side chain metabolites. The chlorinated anilines may be volatilized or bound to soils before or concomitantly with microbial metabolism. It is not clear what effects these competing processes have on biodegradation of these molecules. There have been few studies on the persistence or metabolism of compounds arising from the side chain, particularly those containing chloride ions.

SECTION 13
CHLORINATED BIPHENYLS

Polychlorinated biphenyls (PCBs) have been used widely in industrial applications because of their thermal stability, excellent dielectric (electrically insulating) properties, and resistance to oxidation, acids, bases, and other chemical agents. PCBs therefore have found use in capacitors and transformers as dielectric fluids, in hydraulic systems, gas turbines and vacuum pumps, and as fire retardants and plasticizers (215). In 1971, however, Monsanto Company, the sole U.S. producer, voluntarily restricted the use of PCBs to closed systems (capacitors, transformers, vacuum pumps, gas-transmission turbines) and discontinued production entirely in 1978 (281). These applications use complex mixtures of PCBs marketed under the trade names Aroclor (Monsanto Company, USA), Clophen (Germany), Phenoclor and Pyralene (France), Kaneclor and Santotherm (Japan), and Fenclor (Italy). Askarels are synthetic mixtures of chlorinated biphenyls and trichlorobenzenes.

The Aroclor products are denoted by a four-digit number in which the first two indicate the type of molecule ("12" indicates a chlorinated biphenyl) and the last two digits indicate the weight percent chlorine. Aroclor 1254 consists of chlorinated biphenyls with 54% by weight chlorine and on average 5 chlorines per molecule, although it has been reported to contain 69 different chlorinated biphenyl molecules (162). Similarly, Aroclor 1242 is 42% chlorine by weight and averages 3 chlorines per molecule. Aroclor 1016 also consists primarily of trichlorinated compounds but contains fewer penta- and hexachlorinated molecules than Aroclor 1242. There are 210 possible PCB compounds containing 0 to 10 chlorine atoms per biphenyl molecule. However, many of these have never been found in commercial PCB mixtures.

The PCBs have been released into the environment for many years and are a worldwide contaminant. They are lipophilic and sorb strongly to the lipids and fats of animals including fish, mussels, and birds. PCBs also undergo biological magnification in such common aquatic invertebrates as daphnids, mosquito larvae, stoneflies and crayfish. The concentration of PCBs in the invertebrates can be as high as 27,500 times that in water (162). These invertebrates subsequently are eaten by fish and birds, and bioaccumulation occurs at all levels of the food chain.

MICROBIAL METABOLISM OF PCBs

Most of the studies on microbial metabolism of PCBs have explored the biodegradability of the Aroclors in natural environments or in laboratories using pure strains or mixed cultures. These studies have shown that PCBs containing fewer than 5 chlorines per molecule are extensively degraded, while heavier molecules tend to persist in the environment (26, 91, 163, 167, 220a, 281, 394, 438, 474). These studies are corroborated by environmental analyses which indicate that PCBs found in weathered samples contain 5 or more chlorine atoms per molecule (162).

Few studies have attempted to elucidate the pathways of degradation of pure compounds of a chlorinated biphenyl. Several studies have shown that 4-chlorobiphenyl can be metabolized to 4-chlorobenzoic acid, indicating hydroxylation, ring cleavage, and degradation of the nonchlorinated ring of the molecule. This has been demonstrated with soil bacteria, a sewage effluent isolate identified as *Achromobacter* sp. pCB, an unidentified facultative anaerobe called strain B206, *Acinetobacter* sp. P6, and *Alcaligenes* sp. Y42 (1, 163, 164, 333, 426). *Acinetobacter* sp. P6 can use 4-chlorobiphenyl as the sole source of carbon for growth (164). Growth of both *Achromobacter* sp. strain B 218 and *Bacillus brevis* strain B 257 on 4-chlorobiphenyl as the sole source of carbon generates the same metabolites (298). The pathway of degradation involves formation of a 2,3-dihydroxy intermediate with *meta* cleavage to form eventually 4-chlorobenzoic acid. Other metabolites were isolated which represent successive oxidation and utilization of the aliphatic carbons from the cleaved ring (Figure 56).

The formation of chlorinated benzoic acids from chlorinated biphenyls is the most common route of PCB degradation. Both *Alcaligenes* sp. Y42 and *Acinetobacter* sp. P6 convert a large number of biphenyl compounds to the corresponding chlorinated benzoic acids (Table 5) (163, 168). For several compounds containing multiple chlorines with one on the second ring, loss of that chlorine occurs in the formation of the chlorobenzoic acid. Studies with ^{14}C-2,5,2′-trichlorobiphenyl confirmed the formation of ^{14}C-2,5-dichlorobenzoic acid and a yellow intermediate by resting cell suspensions of both *Alcaligenes* sp. Y42 grown on biphenyl and *Acinetobacter* sp. P6 grown on 4-chlorobiphenyl (164). The patterns of metabolism of PCBs by *Alcaligenes* sp. Y42 and *Acinetobacter* sp. P6 are similar. The general path of degradation proceeds through *meta* cleavage compounds to chlorobenzoic acids which accumulate during the metabolism of chlorobiphenyls. Metabolism of some chlorinated biphenyls is blocked after production of the dihydroxy intermediate (precursor to ring cleavage), while for other compounds the *meta*-cleavage intermediate accumulates.

Pseudomonas sp. strain 7509 grown on biphenyl metabolizes 2,4′-dichlorobiphenyl to two different monochlorobenzoates, indicating that both rings are capable of being attacked (26a). An intermediate metabolite was identified as 2-hydroxy-6-oxo-6-(chlorophenyl)chlorohexa-2,4-dienoic acid.

The degradation of 2,4,4′-trichlorobiphenyl by *Acinetobacter* sp. P6 grown on 4-chlorobiphenyl was studied in detail and a pathway was proposed involving *meta* cleavage after 2′,3′-hydroxylation (Figure 57) (166). Hydroxylation occurs on the ring with the fewest chlorine substituents. The predominant metabolite is the *meta* cleavage product, although small amounts of the dichlorobenzoic acid appear. The occurrence of a yellow *meta* cleavage product and subsequent production of chlorobenzoates in the degradation of other PCBs suggests that this may be a general pathway for most PCB metabolism in bacteria.

On the basis of these studies, several generalizations were made regarding the effect of the structure of the PCBs on microbial degradation (163, 164, 165, 168). Degradation decreases as the number of chlorines per molecule increases. Two chlorines on the *ortho* positions of a single ring (i.e., 2,6-) or on both rings (i.e., 2,2′-) inhibit degradation. PCBs with one unsubstituted ring are more readily metabolized than PCBs with the same number of chlorines on both rings. On PCBs with unequally substituted rings, the ring with fewer substitutions is preferentially cleaved. PCBs with a chlorine on the 4′-position are metabolized to stable *meta*-cleavage products.

The occurrence of nitro-containing metabolites in extracts of media containing the unidentified facultative anaerobe B 206 and 4-chlorobiphenyl was investigated (427). When ammonium sulfate is added to the culture medium as the nitrogen source, 4-chlorobiphenyl is metabolized to 2-hydroxy-4′-chlorobiphenyl and 4-hydroxy-4′-chlorobiphenyl. However, when the nitrogen source is sodium nitrate, the metabolites 2-hydroxy-nitro-4′-chlorobiphenyl and 4-hydroxynitro-4′-chlorobiphenyl ap-

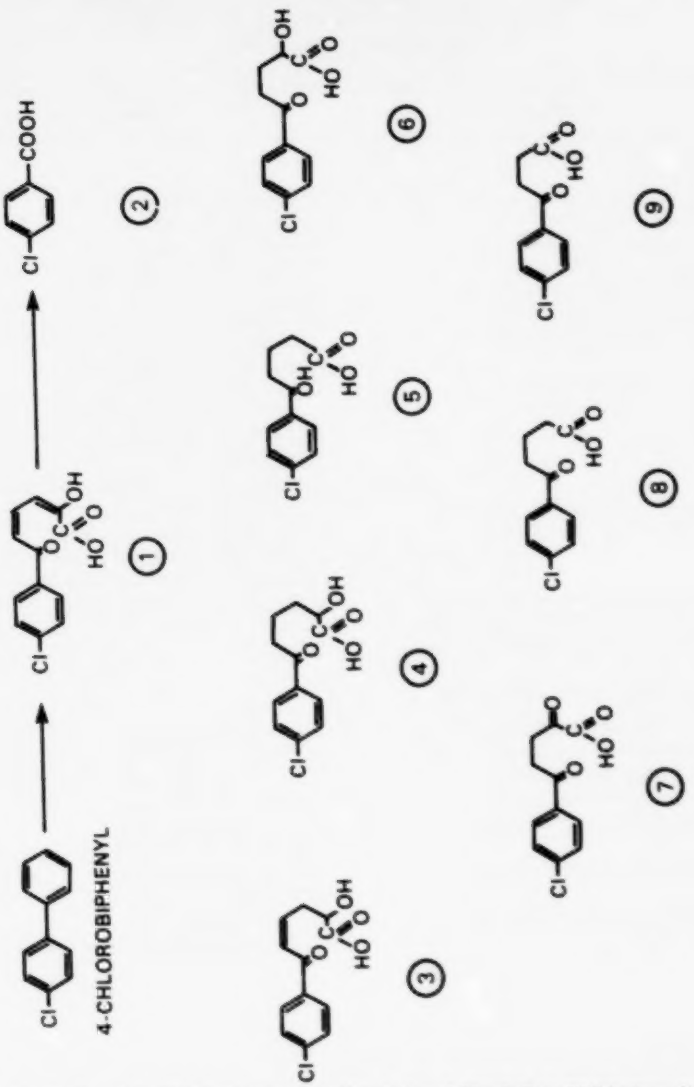


Figure 56. Microbial degradation of 4-chlorobiphenyl. Metabolites: (1) 2-hydroxy-6-oxo-6-(4′-chlorophenyl)-hexa-2,4-dienoic acid; (2) 4-chlorobenzoic acid; (3) 2-hydroxy-6-oxo-6-(4′-chlorophenyl)-4-hexanoic acid; (4) 6-oxo-6-(4′-chlorophenyl)-2-hydroxy-hexanoic acid; (5) 6-hydroxy-6-(4′-chlorophenyl) hexanoic acid; (6) 2-hydroxy-5-oxo-5-(4′-chlorophenyl) pentanoic acid; (7) 2,5-oxo-5-(4′-chlorophenyl) pentanoic acid; (8) 5-oxo-5-(4′-chlorophenyl) pentanoic acid; (9) 4-oxo-4-(4′-chlorophenyl) butanoic acid.

Adapted from Reference 298.

TABLE 5. METABOLISM OF CHLORINATED BIPHENYL COMPOUNDS BY ALCALIGENES SP. Y42 AND ACINETOBACTER SP. P6[*]

Substrate	Products
Substitutions on one ring:	
2-Chlorobiphenyl	2-Chlorobenzoic acid
3-Chlorobiphenyl	3-Chlorobenzoic acid
4-Chlorobiphenyl	4-Chlorobenzoic acid
2,3-Dichlorobiphenyl	2,3-Dichlorobenzoic acid
2,4-Dichlorobiphenyl	2,4-Dichlorobenzoic acid
2,5-Dichlorobiphenyl	2,5-Dichlorobenzoic acid
2,6-Dichlorobiphenyl[‡]	2,5-Dichlorodihydroxybiphenyl, 2,6-dichlorotrihydroxybiphenyl
3,4-Dichlorobiphenyl	3,4-Dichlorobenzoic acid
3,5-Dichlorobiphenyl	3,5-Dichlorobenzoic acid
2,3,4-Trichlorobiphenyl	2,3,4-Trichlorobenzoic acid
2,3,6-Trichlorobiphenyl[‡]	2,3,6-Trichlorodihydroxybiphenyl, 2,3,6-dichlorotrihydroxybiphenyl
2,4,5-Trichlorobiphenyl	2,4,5-Trichlorobenzoic acid
2,4,6-Trichlorobiphenyl	2,4,6-Trichlorobenzoic acid[†], 2,4,6- trichlorodihydroxybiphenyl,[†] 2,4,6-trichlorotrihydroxybiphenyl[‡]
2,3,4,5-Tetrachlorobiphenyl	2,3,4,5-Tetrachlorobenzoic acid
2,3,5,6-Tetrachlorobiphenyl	None
2,3,4,5,6-Pentachlorobiphenyl	None
Substitutions on both rings:	
2,2′-Dichlorobiphenyl	2-Chlorobenzoic acid
2,4′-Dichlorobiphenyl	2-Chlorobenzoic acid
3,3′-Dichlorobiphenyl	3-Chlorobenzoic acid
4,4′-Dichlorobiphenyl	4-Chlorobenzoic acid
2,4,4′-Trichlorobiphenyl	2-Chlorobenzoic acid, 2,4-dichlorobenzoic acid
2,5,2′-Trichlorobiphenyl	2-Chlorobenzoic acid, 2,5-dichlorobenzoic acid
2,5,3′-Trichlorobiphenyl	3-Chlorobenzoic acid, 2,5-dichlorobenzoic acid

(continued)

TABLE 5. (continued)

2,5,4'-Trichlorobiphenyl	2-Chlorobenzoic acid, 2,5-dichlorobenzoic acid
3,4,2'-Trichlorobiphenyl	2-Chlorobenzoic acid
2,3,2',3'-Tetrachlorobiphenyl	2,3-Dichlorobenzoic acid, an unidentified dichloro compund
2,3,2',5'-Tetrachlorobiphenyl	Dichlorobenzoic acid, an unidentified dichloro compound
2,4,2',4'-Tetrachlorobiphenyl+	2,4-Dichlorobenzoic acid
2,4,2',5'-Tetrachlorobiphenyl+	Dichlorobenzoic acid
2,4,3',4'-Tetrachlorobiphenyl+	Dichlorobenzoic acid
2,5,2',5'-Tetrachlorobiphenyl+	2,5,2',5'-Tetrachlorodihydroxyl-biphenyl
2,5,3',4'-Tetrachlorobiphenyl+	Dichlorobenzoic acid
2,6,2',6'-Tetrachlorobiphenyl+	None
3,4,3',4'-Tetrachlorobiphenyl+	3,4-Dichlorobenzoic acid
2,4,5,2',3'-Pentachlorobiphenyl+	2,4,5-Trichlorobenzoic acid, an unidentified trichloro compound
2,4,5,2',5'-Pentachlorobiphenyl+	2,4,5,2',3'-Tetrachlorodihydroxy-benzoic acid

*Adapted from reference 163, 164, 168.

+Metabolism by *Acinetobacter* sp. P6 only.

†Metabolism by *Alcaligenes* sp. Y42 only.

pear. These metabolites were interpreted to result from a nonenzymatic reaction between an arene oxide intermediate and nitrate or nitrite anions. This organism subsequently accumulates 4-chlorobenzoic acid in the medium (426). The appearance of the monohydroxy intermediates suggests a rare monooxygenase mechanism for PCB degradation, although the phenylphenols may also be an artifact arising during the isolation procedure (71a).

Acinetobacter sp. strain P6 resting cells were incubated for 4 hours with several Kaneclor PCB mixtures (167). Kaneclor KC200 (primarily dichlorobiphenyls) was metabolized to monochlorobenzoates. Kaneclor KC300 was metabolized to benzoates with 1 to 3 chlorines, dihydroxybiphenyls with 2 to 4 chlorines, ring *meta* cleavage products with 2 to 3 chlorines, and many other unidentified compounds with 2 chlorines. Kaneclor KC500 was scarcely metabolized, although some dihydroxy isomers were noted.

Studies were conducted utilizing *Alcaligenes* sp. strain BM 2 which was isolated on diphenylmethane and known to metabolize dichlorinated biphenyls (474). A mixture of di- and trichlorinated biphenyls at 0.05% concentration (32% by weight) was 80% metabolized in 1 day and completely metabolized in 3 days. At 0.25% concentration, 22% was metabolized in 1 day and 29% in 3 days. Under cometabolic conditions, a 100 mg/l PCB mixture of di-, tri-, and tetrachlorobiphenyls (41% by weight) was 70% metabolized in 2 days and 80% in 6 days. In a minimal medium with only a small quantity of carbon source, 30% was metabolized in 6 days. Most of the remaining substrate was tetrachlorobiphenyl. The metabolites included mono- di-, and trichlorobenzoates, monohydroxychlorobiphenyl, 2-hydroxy-6-oxo-chlorophenyl-hexa-2, 4-dienoic acid, chlorobenzoylpropionic acid, chlorophenylacetic acid, and 3-chlorophenyl-2-chloropropenoic acid (a substituted cinnamic acid).

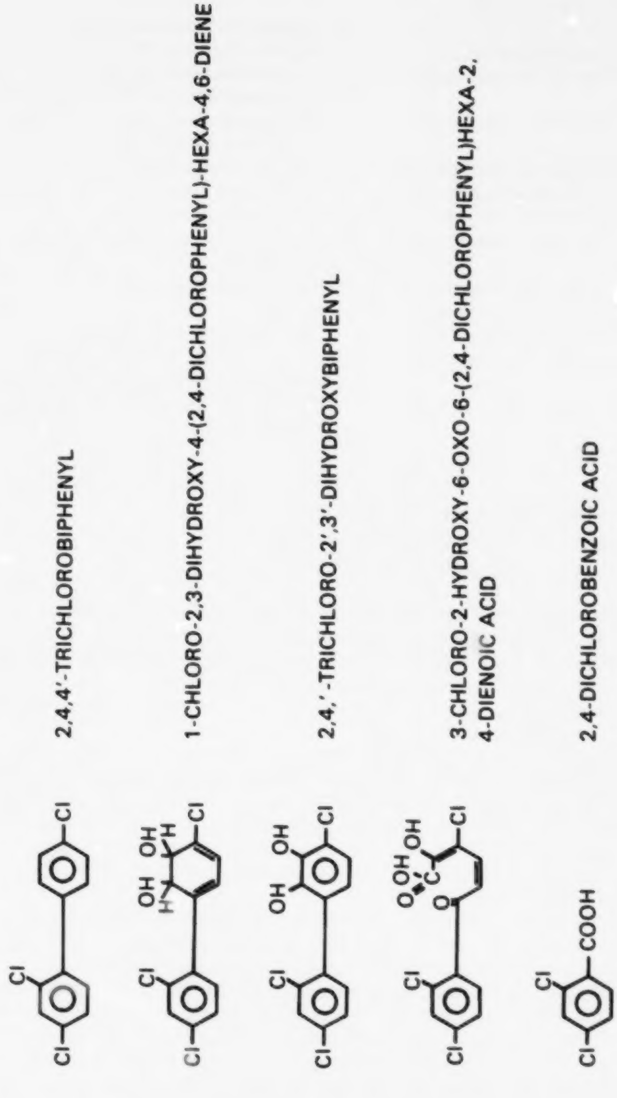

2,4,4'-TRICHLOROBIPHENYL
1-CHLORO-2,3-DIHYDROXY-4-(2,4-DICHLOROPHENYL)-HEXA-4,6-DIENE
2,4,' -TRICHLORO-2',3'-DIHYDROXYBIPHENYL
3-CHLORO-2-HYDROXY-6-OXO-6-(2,4-DICHLOROPHENYL)HEXA-2,
4-DIENOIC ACID
2,4-DICHLOROBENZOIC ACID
Cl
OH
H
COOH

Figure 57. Pathway of 2,4,4'-trichlorobiphenyl metabolism by *Acinetobacter* sp. P6.

Adapted from Reference 166.

Pseudomonas sp. strain 7509 also formed mono- and dichlorobenzoic acids during metabolism of Aroclor 1242 (26a). A number of nonchlorinated aromatic and aliphatic compounds were isolated after 2 months' incubation of Aroclor 1242 with several strains of bacteria isolated from lake water (233). No chlorinated metabolites or oxidized derivatives of PCBs were detected.

The disappearance of Aroclor products and individual PCB isomers during incubation with *Nocardia* sp. NCIB 10603 was monitored (26). The following isomers were 60 to 100% metabolized within 2 weeks: 2,4′-di, 2,3-di, 3,4-di, 2,3,2′-tri, 2,3,4′-tri- and 3,4,3′-trichlorobiphenyl, while 2,5,4′-trichlorobiphenyl was 60% metabolized in 73 days. There was little transformation of 2,4,6-tri-, 2,4,2′,4′-tetra-, or 2,4,6,2′-tetrachlorobiphenyl in 9 days, and no degradation of 4,4′-dichlorobiphenyl was detected after 121 days. However, 4,4′-dichlorobiphenyl was 50% metabolized in 2 days when present as a component of Aroclor 1242. During 52 days' incubation Aroclor 1242 was 88% metabolized and in 100 days was 95% metabolized. Aroclor 1016 was 96% metabolized in 52 days.

There are two reports in the literature concerning the degradation of PCBs by fungi. *Rhizopus japonicus* converts 4-chlorobiphenyl to 4-chloro-4′-hydroxybiphenyl and 4,4′-dichlorobiphenyl to an unidentified hydroxylated metabolite (454). *Cunninghamella echinulata* Thaxter metabolizes 2,5-dichloro-4′-isopropylbiphenyl by oxidation of the isopropyl group to form 2,5-dichloro-4′-biphenylcarboxylic acid and by hydroxylation of the chlorine-substituted phenyl group (440).

METABOLISM OF PCBs BY MIXED MICROBIAL CULTURES

A mixed microbial population in lake water metabolized 2-chlorobiphenyl to 2-chlorobenzoic acid and chlorobenzoylformic acid (394). In contrast, 2,4′-dichlorobiphenyl was not metabolized after 8 months' incubation.

A mixed microbial culture derived from river sediments was able to metabolize 4-chlorobiphenyl rapidly, with 99% removal in 30 days (265). Acclimated and nonacclimated cultures showed similar results. There was transitory formation of a metabolite thought to be 2-hydroxy-6-oxo-6-(4-chlorophenyl)hexa-2,4-dienoic acid as well as production of 4-chlorobenzoic acid. The substrate was ^{14}C-labeled on the chlorinated ring only and production of $^{14}CO_2$ was noted after 4-chlorobenzoic acid formation. The substrates 2-chloro- and 3-chlorobiphenyl were also metabolized, but 2-chlorobenzoic acid was not degraded further.

A marine mixed microbial community metabolized all three monochlorinated biphenyls (52). Metabolites were not identified.

A river water die-away test demonstrated 50% removal of 1 to 100 mg/l 2-chloro-, 3-chloro-, or 4-chlorobiphenyl within 2 to 5 days (17). The compound was uniformly ^{14}C-labeled in the chlorinated ring and up to 50% of the label appeared as monochlorobenzoic acid and subsequently as $^{14}CO_2$. No degradation of 2,2′,4,4′-tetrachlorobiphenyl was noted after 98 days' incubation.

A mixture of bacteria was isolated by enrichment culture with garden soil using benzene as the substrate (18). Several PCB isomers were incubated with the mixed culture and benzene for up to 6 weeks and the medium subsequently tested for presence of chlorobenzoate metabolites. Neither 2-chloro- nor 2,2′-dichlorobiphenyl was metabolized to chlorobenzoic acids, although 4-chlorobiphenyl formed 4-chlorobenzoic acid. The substrate 2,4,4′-trichlorobiphenyl formed copious amounts of 4-chloro- and 2,4-dichlorobenzoic acid in the ratio 5:2. Loss of one chloride was noted in the formation of 4-chlorobenzoic acid from 2,4′-dichlorobiphenyl, 2,5-dichlorobenzoic acid from 2,2′,5-trichlorobiphenyl, 4-chlorobenzoic acid and 3,4-dichlorobenzoic acid from 3,4,4′-trichlorobiphenyl, 4-chlorobenzoic acid and 2,4-dichlorobenzoic acid from 2,4,4′-trichlorobiphenyl, and 2,3,4,5-tetrachlorobenzoic acid from both 2,3,3′,4,5-pentachlorobiphenyl and 2,3,4,,4′,5-pentachlorobiphenyl.

A soil plot was treated with 1 ppm 2,2′-dichlorobiphenyl (317). After one growing season almost half the remaining material was unchanged substrate. About 9% were soluble metabolites and almost 42% were unextractable residues. After 1 year, 74% of the remaining material were unextractable residues. The metabolites included monohydroxy derivatives of the substrate as well as other products.

Metabolism of 4,4′-dichlorobiphenyl by a mixed microbial culture obtained from the filtrate of an activated sludge sample resulted in formation of 4,4′-dichloro-2,3-hydroxybiphenyl and 4-chlorobenzoic acid (439). Metabolism of this substrate is repressed by the presence of alternative carbon sources. Under similar experimental conditions, including an incubation time of 14 days, there was no metabolism of 2,4,5′-tri-, 2,2′,5,5′-tetra, 2,2′,3,4,5′-penta-, 2,2′,3,4,5,5′-hexa- and decachlorobiphenyl.

Metabolism of 2,5,2′,5′-tetrachlorobiphenyl and 2,5,2′-trichlorobiphenyl occurred in seawater with production of a compound thought to be a lactone acid (68a). No degradation was noted during the incubation of the tetrachlorobiphenyl with anaerobic marsh mud during a 45-day incubation period.

A microbial consortium obtained from activated sludge metabolized the isopropyl group of 4-chloro-4′-isopropylbiphenyl to a hydroxyl substituent, forming 4-chloro-4′-hydroxybiphenyl, followed by formation of 4-chlorobenzoic acid (440). Intermediates of the isopropyl metabolism pathway were identified. Addition of glucose as an alternate carbon source repressed metabolism of the chlorinated substrate.

Biphenyl enrichment of both uncontaminated soils and soils contaminated with PCBs resulted in isolation of mixed cultures which were incubated with Aroclor 1242 (91). Cometabolism with sodium acetate enhanced metabolism of all the PCBs including the higher chlorinated molecules, although this phenomenon may be due to the increased biomass resulting from growth on the simpler carbon source. Extensive degradation of all the lower chlorinated isomers was noted with up to 68% metabolism of the tetrachlorinated biphenyls in 15 days.

A related study was conducted to determine the influence of inoculum concentration on the aerobic bio-oxidation of 3,3′-dichlorobenzidine which is used in the manufacture of azo dyes (57). The effluent from a domestic sewage treatment plant was used as the inoculum and the substrate concentration was 20 mg/l. When present as the sole carbon source, the substrate was not metabolized. However, the presence of yeast extract in the medium promoted extensive disappearance of the substrate within 28 days. In another experiment, 2 mg/l 3,3′-dichlorobenzidine was added to lake or reservoir water and incubated 14 days (10). Neither metabolites nor $^{14}CO_2$ was recovered after incubation. Increasing disappearance of substrate with time was correlated with increasing biomass, which served as a sorbent for the substrate. The supernatant fluid from settled activated sludge material served as the inoculum for flasks containing 3,3′-dichlorobenzidine and additional carbon sources. After 4 repeated weekly subcultures to flasks of fresh media, no metabolites of the substrate were recovered.

SUMMARY

The limited number of studies on the degradation of specific chlorinated biphenyl compounds by pure strains of bacteria has served to establish some general features of PCB metabolism. A few strains of bacteria have been shown to mineralize some chlorinated biphenyls. In most cases bacteria with the capability of degrading one ring of a chlorinated biphenyl compound are unable to degrade the resulting chlorinated benzoates. These compounds accumulate in pure cultures. Evidence exists for the complete mineralization of chlorinated benzoates by other strains of bacteria (discussed in Section 7 on chlorobenzoates), and mixed cultures of bacteria have been shown to mineralize PCBs with 4 or fewer chlorines per molecule. More heavily substituted PCBs appear to resist degradation and accumulate even in environments where lower PCBs are degraded.

The mechanism of hydroxylation of PCBs by bacteria has not yet been elucidated, nor have the enzymes mediating the steps in the proposed pathways been isolated. Two pathways have been proposed, the first analogous to the pathway of degradation of biphenyl. Initial hydroxylation occurs in the 2,3-position of the less substituted ring, followed by *meta* cleavage and subsequent degradation of the aliphatic portion of the molecule to form substituted benzoic acids. Chlorines on the aliphatic carbons are lost during this process. However, this may not be the mechanism for degradation of PCBs substituted in all the *ortho* positions. A second pathway based on presence of a monooxygenase in bacteria has been proposed after discovery of 4-hydroxy-4'-chlorobiphenyl in extracts of bacterial cultures incubated with 4-chlorobiphenyl. More evidence corroborating this mechanism needs to be obtained to determine how widespread this pathway is. Limited evidence on fungal metabolism of PCBs indicates activity of a monooxygenase in a manner similar to that shown in biphenyl metabolism.

SECTION 14
DDT AND RELATED COMPOUNDS

DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethene, received its abbreviation from the trivial name dichlorodiphenyltrichloroethane. This chlorinated aromatic is one of the most persistent pesticides in the environment. Since it is lipophilic, it readily accumulates in microorganisms and invertebrates and undergoes biomagnification as fish and birds higher in the food chain ingest DDT-contaminated organisms (79, 229, 263, 311). Widespread effects of DDT poisoning include eggshell thinning and birth defects. Reviews of the literature pertaining to DDT metabolism in microbial systems have been published in 1976 and 1980 (138, 228).

BACTERIAL METABOLISM OF DDT

Due to the widespread incidence of toxicity demonstrated after DDT ingestion, the intestinal flora of various animals became the focus for studies of the metabolism of DDT. DDT is converted directly to DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl)-ethane, also referred to as dichlorodiphenyldichloroethane (Figure 58). The reaction involves removal of a chloride ion from the aliphatic portion of the molecule. This is a reductive dechlorination reaction requiring anaerobic conditions, in contrast to the oxidative pathways of metabolism of most other pesticides.

The involvement of intestinal bacteria in DDT metabolism by mammals has been demonstrated in experiments which showed that rats converted DDT to DDD when fed by stomach tube, but not when injected intraperitoneally (308). The coliform bacteria *Escherichia coli* and *Enterobacter aerogenes* isolates from rat feces demonstrate this reaction as well. Microorganisms isolated from rat intestines which convert DDT to DDD include *Clostridium perfringens*, *Streptococcus* sp., *Bacteroides* sp., *E. coli* and other coliforms, yeasts, and to a lesser extent *Lactobacillus* sp. (55). *Klebsiella pneumoniae* also converts DDT to DDD (464). DDE (1,1-bis(*p*-chlorophenyl)-2-chloroethylene) can be detected after 20 hours at concentrations from 5 to 10% in cultures of *Streptococcus* sp., *Bacteroides* sp., *Pseudomonas* sp. and *Lactobacillus* sp. (the latter after a 72 hour incubation) (55). However, DDE has been shown to be produced nonenzymatically as well as enzymatically (464).

Proteus vulgaris, isolated from the intestines of DDT-resistant mice, converts DDT to DDD subsequently to 1,1-bis(*p*-chlorophenyl)-2-chloroethane and 1,1-bis(*p*-chlorophenyl)ethane, representing three successive reductive dechlorinations (Figure 58) (20,21). DDE also appears in the medium. The excreta of stable flies became the source of three bacteria, *E. coli*, *Serratia marcescens*, and a third unidentified strain, which convert DDT to DDD (90%) and DDE (5%) after 24 to 72 hours anaerobically but not aerobically (415). Bacteria from bovine rumen fluid convert ^{14}C-DDT to ^{14}C-DDD (311). This same reaction was noted for DDT incubated with water from Clear Lake, California and with reduced iron porphyrins (hemoglobin or hematin) (311). The isomer *o,p'*-DDT which constitutes 10-20% of

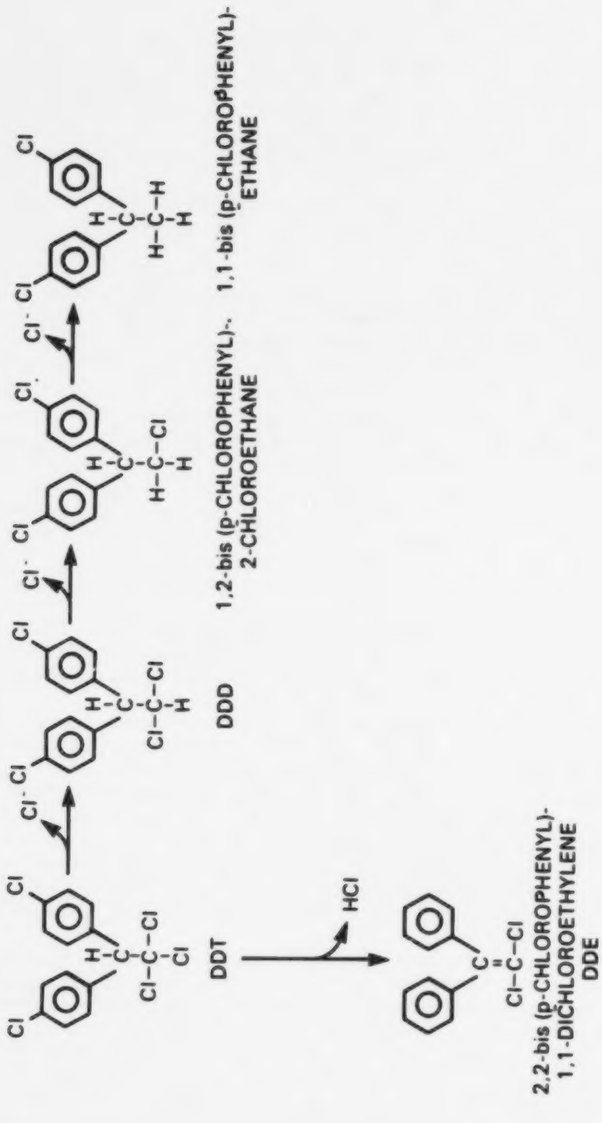

DDT
Cl⁻
DDD
Cl⁻
1,2-bis (p-CHLOROPHENYL)-2-CHLOROETHANE
Cl⁻
1,1-bis (p-CHLOROPHENYL)-ETHANE
HCl
2,2-bis (p-CHLOROPHENYL)-1,1-DICHLOROETHYLENE
DDE

Figure 58. Reductive dechlorination of DDT and dehydrochlorination of DDT.

technical grade DDT is converted by rumen microorganisms to *o,p'*-DDD at the same rates as *p,p'*-DDT (161). This conversion occurs as well in *E. aerogenes* both aerobically and anaerobically (309).

The direct conversion of DDT to DDD without the DDE intermediate was confirmed in *E. aerogenes* using deuterated DDT (355). The deuterium atom present at the 2-position in DDT is retained in the product, indicating that the chlorine is replaced by hydrogen (or hydride ion) directly without the intermediary species. The membranes of *E. coli* were shown to be the site of reductive dechlorination of this species. The process required flavine-adenine dinucleotide (FAD) and anaerobic conditions (160).

Cell-free extracts of *E. aerogenes* also convert DDT to DDD. This activity is due to reduced Fe(II) cytochrome oxidase (464). More complete degradation of DDT occurs in both whole-cell preparations and cell-free extracts of this organism (Figure 59)(463,465). Metabolism follows the pathway DDT → DDD → DDMU → DDMS → DDNU → DDA → DPM → DBH → DBP →, where the abbreviations represent the compounds as follows: (DDMU) 1-chloro-2,2-bis(p-chlorophenyl)ethylene; (DDMS) 1-chloro-2,2-bis(p-chlorophenyl)ethane; (DDNU) unsym-bis(p-chlorophenyl)ethylene; (DDA) 2,2-bis(p-chlorophenyl)acetate or more commonly dichlorodiphenylacetate; (DPM) dichlorodiphenylmethane; (DBH) dichlorobenzhydrol; and (DBP) dichlorobenzophenone. The enzymatic conversion of DDT to DDE is a dead-end side reaction. DDA is the end product of vertebrate metabolism. The conversion of DDA to DBP does not require anaerobic conditions. This pathway has also been demonstrated in anaerobic cultures of *E. coli* (268).

A single study reports that under aerobic conditions, cultures of *Bacillus cereus* metabolize DDT by this pathway within 7 days, although the use of screw-cap flasks in these experiments may have allowed some anaerobiosis to develop. Cultures of *E. coli* incubated aerobically for 24 hours with intermittent shaking converted DDT to DDD (75%) and DDE (25%) (247).

Environmental isolates also have the ability to convert DDT to DDD. Viable cells of *Bacillus megaterium* convert DDT to DDD (201). Three hundred bacterial strains from Lake Michigan each converted DDT to DDD and many converted ^{14}C-DDD to ^{14}C-DDNS (1-bis(*p*-chlorophenyl)ethane (302). Bacteria isolated from marine and brackish water and sediment converted ^{14}C-DDT to water-soluble metabolites (232). Forty-seven of 100 isolates effected 5 to 10% conversion while an additional 38 isolates converted less than 5% of the starting material. Twenty-five isolates did not produce water-soluble metabolites, indirectly indicating that the presence of those metabolites in the other cultures was biologically mediated. Twenty-three of 26 plant pathogenic and saprophytic strains of bacteria representing nine genera, converted DDT to DDD anaerobically (230). Eighteen bacterial strains, mostly *Pseudomonas* spp. which previously had been shown to metabolize dieldrin, also degraded DDT to DDD (349). In addition, 14 of these isolates produced DDA and 10 produced a dicofol-like compound.

Pseudomonas aeruginosa 640x isolated from DDT-polluted soil of the Crimean region was used to construct 2 derivatives (185). Strain BS816 carries a plasmid encoding the genes which degrade naphthalene and salicylate by *ortho* cleavage, and strain BS827 carries a plasmid which effects *meta* degradation. Both plasmids were obtained from strains of *P. putida*. The parent *P. aeruginosa* and the two derivatives all metabolize DDT with the formation of the same metabolites. Strain BS816, carrying the plasmid coding for *ortho* cleavage, degrades DDT most extensively, converting 89% of the DDT to DDD,1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDDE), phenylpropionic acid (PPA) and phenylacetic acid (PAA).

An organism identified as a *Pseudomonas* sp. was isolated by enrichment culture for its ability to use diphenylethane as a sole source of carbon and energy for growth

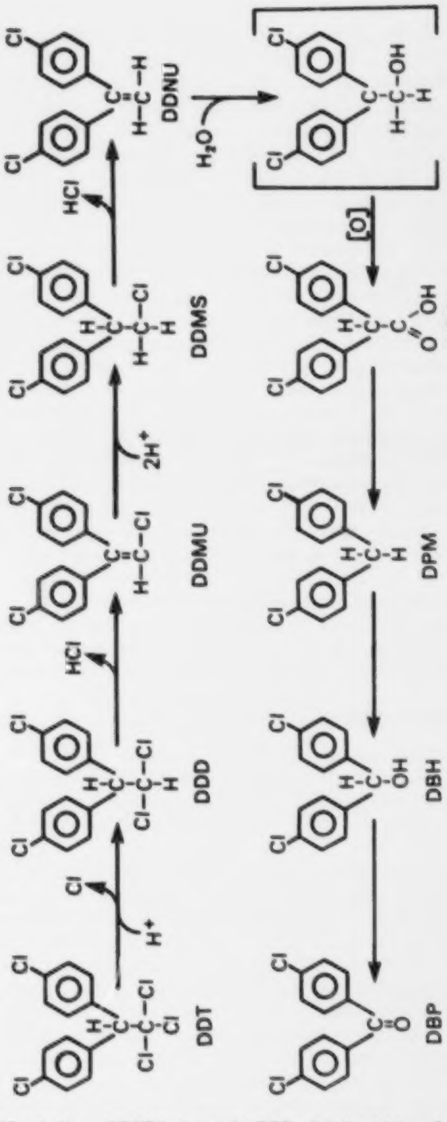

DDT
H⁺
Cl⁻
DDD
HCl
DDMU
2H⁺
DDMS
HCl
DDNU
H₂O
DDOH
[O]
DDA
DPM
DBH
DBP

Figure 59. Metabolism of DDT by bacteria. DDT = 1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane; DDD = 1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethane; 2,2-bis (*p*-chlorophenyl) ethane; DDNU = unsym-bis (*p*-chlorophenyl) ethylene; DDOH = 2,2-bis (*p*-chlorophenyl) ethanol (hypothetical metabolite); DDA = dichlorodiphenylacetate; DPM = dichlorodiphenylmethane; DBH = dichlorobenzhydrol; DPB = dichlorobenzophenone.

Adapted from References 463, 465.

(157). The ability of this organism to grow on several other metabolites of DDT was tested. Diphenylethane is converted to 2-phenylpropionic acid and metabolized further. Diphenylmethane is metabolized with intermediate production of phenylacetic acid and 1-(*p*-chlorophenyl)-1-phenylethane is metabolized with the production of 2-(*p*-chlorophenyl)proprionic acid as the only metabolite, indicating cleavage of the unsubstituted ring. The DDE analog 1-(*p*-chlorophenyl)-1-phenylethene is metabolized only to 2-(*p*-chlorophenyl)-2-propenoic acid. The substrate 1-*p*-chlorophenyl)-1-phenylethanol is converted to 2-(*p*-chlorophenyl)-2-hydroxypropionic acid which is slowly metabolized further. During metabolism of this substrate minor amounts of the nonchlorinated metabolite 2-hydroxy-2-phenylpropionic acid are produced, indicating attack on the chlorinated ring. Each of the above substrates served as the sole source of carbon and energy for growth. The compound 2,2-diphenylethanol is not metabolized. Analogs of DDT which have chlorine substituents on both rings are not metabolized, and 1,1-diphenyl-2,2,2-trichloroethane is not metabolized. However, when diphenylethane is available in the medium, this organism cometabolizes bis(*p*-chlorophenyl)methane to *p*-chlorophenylacetic acid which accumulates (158). With the same cosubstrate, the organism cometabolizes 1,1-bis(*p*-chlorophenyl)ethane to 2-(*p*-chlorophenyl)propionic acid with transient appearance of two hydroxylated metabolites, 1-(*p*-chloro-o-hydroxyphenyl)-1(*p*-chlorophenyl)ethane and 1-(*p*-chloro-m-hydroxyphenyl)-1-(*p*-chlorophenyl)ethane. Accumulation of toxic chlorinated carboxylic acids may have inhibited metabolism of the substrate. Compounds which are recalcitrant to cometabolic activity have substitutions in the ethane or ethene sections of their structures which may cause steric hindrance.

A large sampling study of oceanic and near-shore environments established that 35 of 95 isolates degraded DDT to many of the metabolic products previously identified, with DDD the major metabolite (350). The only environmental samples which failed to mediate DDT degradation were the oceanic water samples.

FUNGAL METABOLISM OF DDT

The earliest studies involving microbial metabolism of DDT were conducted using commercial yeast cakes (*Saccharomyces cerevisiae*) (234). Reductive dechlorination was demonstrated by the appearance in culture media of ^{14}C-DDD from ^{14}C-DDT labeled in the phenyl group. DDE was shown not to be a necessary intermediate metabolite.

A study of 8 fungi incubated with DDT for 6 days did not reveal degradation (80). However, in the same study 6 of 9 actinomycetes were found to convert DDT to DDD. These 6 actinomycetes are *Nocardia erythropolis, Streptomyces aureofaciens, S. viridochromogenes, S. cinnamoneus*, and with lesser efficiency *S. albus* and *S. antibioticus*. All cultures in this study were incubated aerobically with shaking. Another study of microbial cultures with the capability to degrade dieldrin showed that 2 *Trichoderma viride* strains could degrade DDT to DDD, a "dicofol-like" metabolite, and DDA (349). The dicofol-like compound was subsequently identified as 1-bis(*p*-chlorophenyl)ethane (302). Earlier studies with several strains of *T. viride* established differences in the metabolites produced by each strain (300).

Shake cultures of *Mucor alternans* in nutrient media containing ^{14}C-DDT produced 3 hexane soluble and 2 water soluble metabolites within 2 to 4 days (7). The total activity recovered was about equally divided between the two phases. The major metabolite is water soluble. These metabolites were unidentified since the results obtained from thin layer chromatography were different from those for DDE, DDD, DDA, DBP, dicofol, or 1,1-bis(*p*-chlorophenyl)ethane. *M. alternans* converted 15% of the DDT starting material to 3 unidentified water soluble products in another experiment as well (232). A comparison of these products with 1,1-bis(*p*-chlorophenyl)acetic acid (DDA), PCPA, DBP, DBH and 2-chlorosuccinic acid failed to reveal their identities. Attempts to reproduce this metabolic activity in the natural environment were unsuccessful, as the addition of *M. alternans* spores to DDT-treated soil failed to promote any degradation after 11 weeks' incubation (7).

A sequential experiment was developed to study the interactive effects of bacteria and fungi (156). *Hydrogenomonas* sp. was grown on dichlorodiphenylmethane or *p*-chlorophenylacetic acid, both metabolic products of DDT. *Hydrogenomonas* sp. cannot liberate free chloride from metabolism of these compounds. The culture supernatant fluid was extracted and added to a basal salts solution, which became the growth medium for a culture of *Fusarium* sp. Growth occurred under anaerobic conditions, and chloride was detected in the medium, indicating that the products of *Hydrogenomonas* sp. metabolism were degraded to CO_2, H_2O, and HCl by *Fusarium* sp. The ability to perform this mineralization decreased if the 2 microbial populations were incubated together.

In another study of the interactive effect of other fungi on DDT degradation by *M. alternans*, the addition of other fungal cultures or the cell-free spent media from some cultures repressed the formation of water soluble metabolites (6). Other fungi, including *Aspergillus flavus, A. fumigatus, A. niger, Fusarium oxysporum, Penicillium notatum, Rhizopus arrhizus* and *Trichoderma ciride*, failed to produce water soluble metabolites of DDT. However, water soluble ^{14}C-products appeared after incubation of ^{14}C-DDT with the excretory products retained in culture media after growth of all of the above fungi including *M. alternans*, with the exception of *R. arrhizus*. This discrepancy in the appearance of water soluble metabolites may be attributed to sorption of degradation products by the mycelia or to further degradation by cells to metabolites that are not water soluble.

The path of DDT metabolism by *Fusarium oxysporum* has been established and follows the route DDT → DDD → DDMU → DDHO → DDOH → DDA → DBP, with DDE formed from DDT (135, 136, 136a). DDHO is the aldehyde intermediate which is rapidly converted to DDOH and DDA. This path is similar to the described for bacteria. The enzymes involved in DDT metabolism which have been isolated include DDT dehydrochlorinase and those that decompose DDMU, DDA, and DDOH (165a). DDT inhibits the fungal esterase while DDD strongly activates the same enzyme (159). The net effect is enzyme activation which results in detoxification of the molecule.

FUNGAL METABOLISM OF OTHER COMPOUNDS

The acaricide chlorobenzilate (ethyl 4,4'-dichlorobenzilate) ^{14}C-labeled in the aliphatic moiety was cometabolized by *Rhodotorula gracilis* with glucose as an additional carbon source (312, 313). Production of $^{14}CO_2$ was correlated with culture growth. Metabolites included 4,4'-dichlorobenzilic acid, dichlorobenzophenone, and other unidentified products. The same results were obtained in the metabolism of chloropropylate (isopropyl 4,4'-dichlorobenzilate). Alteration of the chlorinated rings was not noted.

PERSISTENCE AND DEGRADATION OF DDT IN THE ENVIRONMENT

DDT is converted to DDD by anaerobic but not aerobic sludge microorganisms (202). The same results were found using Pawnee silt loam treated with ^{14}C-DDT. Under anaerobic conditions DDD was recovered while under aerobic conditions DDE was the only metabolite (190). Another study of anaerobic soil treated with

DDT reported formation of DDD and traces of other metabolites including DDE, DDA, dicofol, 4-chlorobenzoate, DBP, and DBM (189a). Conversion of DDT to DDD in flooded soil was faster when more organic matter was present (69).

The conversion of DDT to DDD has been demonstrated in sterile as well as nonsterile environments (59), and has been related to the redox potential (Eh) of the soil. Sewage sludge samples sterilized in a variety of ways all resulted in conversion of DDT to DDD if the Eh was sufficiently low (489). The rate of DDT degradation is highest in soils with the lowest redox potentials, in the range of -90 to -250 mV (184). Studies of DDT degradation in a variety of anaerobic and aerobic environments using various carbon sources and various soils, have shown differences in the amount of degradation and the efficiency of substrate and product recovery which preclude extrapolation from laboratory to the environment or even from one experiment to another (346, 347). Little conversion was found in moist anaerobic soil with Eh of +350 mV or in flooded anaerobic soil with an Eh that dropped from +400 to +200 mV. Flooded stirred soil (Eh = 0 m V) also showed little degradation. However, stirred anaerobic soil treated with lime (Eh = -250 m V) and glass beads inoculated with muck (Eh = -250 to -300 m V) mediated greater than 95% conversion of DDT (346).

It appears that at low Eh, DDT undergoes an irreversible redox type of reaction with transient formation of a free radical before conversion to DDD (184). The reaction is thought to be mediated by reduced iron porphyrins, with cell metabolism not being necessary (489).

Numerous studies have been conducted with regard to the persistence of DDT and its metabolites in environments treated with the insecticide (115, 121, 278, 324, 344, 429, 444, 485). All of the studies have reported residues of DDT, DDE and DDD in the environment at the time of sampling. The longest period of time between last application of DDT and sampling was 17 years, and on the basis of these data half-life numbers for DDT of 2.5 to 35 years have been reported (324). In general, the amount of these residues that remains in the soil 1 to 2 years after application is similar to the amount recovered 9 years or longer after the first sampling.

SUMMARY

Studies on metabolism of DDT by bacteria and fungi have shown that reductive dechlorination of the nonaromatic portion of the molecule is the necessary primary step. The pathway of metabolism of DDT, first described for *E. aerogenes* but subsequently confirmed in other bacteria and in fungi, describes a series of steps requiring anaerobiosis (DDT to DDA) followed by a series of steps that may require aerobic conditions (DDA to DBP). Although some studies report aerobic conversion of DDT to metabolites, the oxygen tension was not rigorously defined in these experiments. All of these metabolites retain the chloride ions on both aromatic rings of the molecule.

The effect of a concerted attack by several microbial species has been demonstrated in a two-stage experiment in which *Hydrogenomonas* sp. was grown on either of the DDT metabolized dichlorodiphenylmethane or *p*-chlorophenylacetic acid. The resulting filtered media became the growth substrate for cultures of the fungus *Fusarium* sp., which anaerobically liberated free chloride, indicating mineralization to CO_2, H_2O, and HCl. The nature of this pathway and the enzymes involved have not yet been elucidated.

Other studies which have shown differing efficiencies of DDT metabolism between pure culture and consortia or sewage/soil studies, indicate that degradation of this compound is highly dependent on environmental factors including coexistence of other organisms. DDT persists in the environment despite the presence of organisms capable of metabolizing the compound to at least DDD. Of particular importance is the redox potential of the environment. Only under highly reducing conditions can the necessary first step of conversion of DDT to DDD be achieved. This reaction does not require microbial mediation. Further steps in DDT degradation may require environmental conditions and microbial activities which have not yet been elucidated. DDT, DDD, and DDE are highly persistent in all environments treated with DDT.

SECTION 15
CHLORINATED DIOXINS AND DIBENZOFURANS

The chlorinated dibenzo-*p*-dioxins and dibenzofurans are produced as byproducts during the formation of many other chemicals, including 2,4,5-T, hexachlorophene, pentachlorophenol and other chlorinated phenols, and polychlorinated biphenyls (360). Chlorinated dioxins have been found in the fly ash and flue gases from municipal generators in Switzerland, presumably due to pyrolysis of chlorophenol salts, and the formation of chlorinated furans has been tied to the pyrolysis of polychlorinated biphenyls and polychlorinated diphenyl ethers. These compounds are used as heat exchange fluids and as hydraulic liquids. From 3 to 25% of the polychlorinated biphenyls burned may be converted to chlorinated dibenzofurans (360). There is no known technical use for the chlorinated dibenzo-*p*-dioxins, of which 75 congeners can exist, and the chlorinated dibenzofurans, of which there are 135 theoretical congeners (360). The positional isomers of the dioxins vary greatly in their acute toxicity and biological activity, and the most potent isomer, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), is considered the most potent low-molecular-weight toxin known (mean lethal dose in guinea pigs 0.6 mg/kg body weight) (387).

Interest in these compounds was generated after an epidemic of "chick edema factor" in 1957 due to 1,2,3,7,8,9-hexachlorodibenzo-*p*-dioxin that caused the death of millions of broiler chickens, and an accident in a chemical plant in 1976 in Seveso, Italy that released a cloud of toxic materials including TCDD to the surrounding environment (259, 356). TCDD has also been shown to cause chick edema factor (387). The extreme toxicity of the compound of major interest, TCDD, has focused most research on this isomer.

MICROBIAL METABOLISM OF DIOXINS AND FURANS

To date none of the chlorinated or nonchlorinated dioxins or furans have served as a sole source of carbon or energy for growth by any microorganism in a wide range of screening and enrichment experiments (214, 259, 260, 301). *Pseudomonas* sp. NCIB 9816, which can utilize naphthalene as a sole carbon source, can cometabolize the nonchlorinated molecule dibenzo-*p*-dioxin when salicylic acid is present in the growth medium (259). Studies to determine the products of cometabolism were conducted with a mutant, *Pseudomonas* sp. NCIB 9816 strain 11, which oxidizes naphthalene only to *cis*-1,2-dihydroxy-1,2-dihydronaphthalene. Dibenzo-*p*-dioxin is cometabolized to 2 neutral products, identified as *cis*-1,2-dihydroxy-1,2-dihydrodibenzo-*p*-dioxin and 2-hydroxydibenzo-*p*-dioxin. When the first product is incubated aerobically or anaerobically with cell extracts of the parent organism in a medium containing NAD+, a third product is formed and was identified as 1,2-dihydroxydibenzo-*p*-dioxin (Figure 60). This metabolite completely inhibits or inactivates the enzyme 1,2-dihydroxynaphthalene oxygenase, which in the parent splits the naphthalene ring in the analogous pathway.

Similarly, a *Beijerinckia* sp. grown on dibenzo-*p*-dioxin and succinic acid produces 1,2-dihydroxydibenzo-*p*-dioxin (260). Cell growth is inhibited after 4

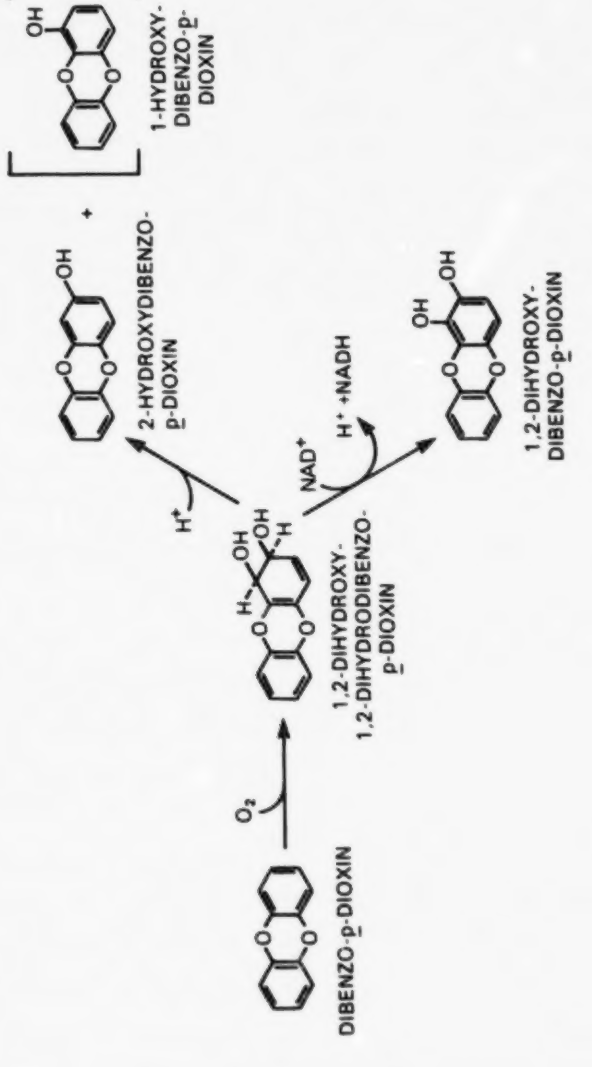

DIBENZO-p-DIOXIN
O2
1,2-DIHYDROXY-1,2-DIHYDRODIBENZO-p-DIOXIN
H+
2-HYDROXYDIBENZO-p-DIOXIN
+
1-HYDROXY-DIBENZO-p-DIOXIN
NAD+
H+ +NADH
1,2-DIHYDROXY-DIBENZO-p-DIOXIN
OH
H

Figure 60. Oxidation of dibenzo-*p*-dioxin by *Pseudomonas* sp. NCIB 9816. Bracketed compound is hypothetical intermediate.

Adapted from Reference 259.

hours. Cell extracts incubated with 1,2-dihydroxydibenzo-*p*-dioxin show a brief initial rate of oxidation followed by a decline to the nonenzymatic rate. The rate of oxidation was determined polarographically using an oxygen electrode to measure oxygen consumption. Two oxygenases were isolated from the cell extract, 2,3-dihydroxybiphenyl oxygenase which also oxidizes 1,2-dihydroxydibenzo-*p*-dioxin, and catechol oxygenase which has no activity against the dioxin metabolite. Both these oxygenases are inhibited when incubated with cell extracts.

This *Beijerinckia* sp. utilizes biphenyl as a sole carbon and energy source. When grown on succinic acid plus biphenyl, resting cells oxidize 1-chloro- and 2-chlorodibenzo-*p*-dioxin at a high rate. Dibenzo-*p*-dioxin and the isomers 2,3-, 2,7-, and 2,8-dichlorodibenzo-*p*-dioxin are oxidized at a lower rate, followed by 1,2,4-trichlorodibenzo-*p*-dioxin (260).

A mutant strain of this species called *Beijerinckia* sp. B8/36 was isolated which metabolizes several aromatic hydrocarbons to *cis*-dihydrodiols (260). When grown on succinic acid plus dibenzo-*p*-dioxin, a neutral product is formed which was identified as *cis*-1,2-dihydroxy-1,2-dihydrodibenzo-*p*-dioxin and has identical characteristics to the product of *Pseudomonas* sp. NCIB 9816 metabolism. Also appearing in the medium is the metabolite 2-hydroxydibenzo-*p*-dioxin. The mutant *Beijerinckia* sp. B8/36 cometabolizes 1-chlor- and 2-chlorodibenzo-*p*-dioxin to neutral products which appear to be *cis*-dihydrodiols, but no products appear after cometabolism with 2,3-dichloro- or 2,7-dichlorodibenzo-*p*-dioxin.

An unidentified bacterium was isolated from contaminated Seveso soil and incubated aerobically in a complex nutrient medium containing ^{14}C-TCDD (214, 351, 352). After 54 weeks 2 polar metabolites appeared. One was isolated in very small quantities and was not identified. The other was found to be a hydroxylated derivative. This microbial metabolite also appears in a culture of *P. testosteroni* strain G1036 after incubation for 36 weeks and in a culture composed of a mix of 6 bacteria from Seveso soil. The metabolite was postulated to be 1-hydroxy-2,3,7,8-TCDD, assuming no chlorine rearrangement took place.

TCDD was metabolized by *Bacillus megaterium* to several polar metabolites (358). The most active cultures were incubated with 5 μg/l TCDD introduced in an ethyl acetate carrier, a solvent which increases cell permeability. When ethyl acetate was the carrier and the amount of soybean extract in the medium was reduced, as much as 55% of the dioxin was recovered as polar metabolites. TCDD was also converted in small amounts to a polar metabolite in farm soil which had been incubated for 2 months. The quantity of this metabolite did not increase with time after 2 months. Other soils similarly incubated failed to produce any metabolites. Two strains of bacteria which converted TCDD to polar metabolites were isolated from the farm soil samples.

A large screening study examined 100 bacterial isolates for ability to metabolize TCDD (301). These strains all had shown previous ability to metabolize persistent pesticides, but only 5 showed some ability to metabolize TCDD as determined by thin-layer chromatography. The product or products were not identified.

Degradation of TCDD by an extracellular laccase (*p*-diphenol:oxygen oxidoreductase) produced by the fungus *Polyporus versicolor* was investigated (68). Crude enzyme extracts incubated with TCDD under a variety of conditions failed to modify the substrate.

There has been one report on the microbial metabolism of dibenzofuran (77). A comparison was made of the cooxidation of this compound by *Cunninghamella elegans*, *Beijerinckia* sp. and *Beijerinckia* sp. B8/36 discussed previously with regard to dibenzo-*p*-dioxin metabolism. The mutant strain oxidizes dibenzofuran to a mixture of 1,2-dihydroxy-1,2-dihydrodibenzofuran and the unstable 2,3-dihydroxy-2,3-dihydrodibenzofuran which under acidic conditions dehydrates to a mixture of

2-hydroxy- and 3-hydroxydibenzofuran (Figure 61). The fungal culture forms a much more stable 2,3-dihydrodiol which yields 2-hydroxydibenzofuran and 3-hydroxydibenzofuran only when heated with acid, although the ratios of the 2 products are similar after bacterial or fungal metabolism. These results are consistent with the unstable bacterial metabolite being of the *cis* configuration and the stable fungal metabolite arising from an epoxide to form a *trans* configuration. The fungal culture also forms a small amount of 2,3-dihydroxydibenzofuran, and the parent *Beijerinckia* sp. in the presence of NAD+ forms 1,2-dihydroxy- and 2,3-dihydroxydibenzofuran.

DIOXIN PERSISTENCE AND DEGRADATION IN SOILS

Evaluation of the persistence of TCDD in soils is complicated by the strong sorptive properties of TCDD, making recovery for analysis difficult (301, 352). In addition, artifacts may arise during the exhaustive extraction and analytical procedures involved. TCDD incubated with lake sediment for less than 1 hour and then extracted with solvents and analyzed by thin layer chromatography and ^{14}C-radioactivity showed 6 to 7% conversion by metabolism to metabolites, indicating the generation of artifacts during the procedure or the presence of impurities (458). Analysis of a commercial ^{14}C-TCDD preparation revealed the presence of 7% contaminants, including TriCDD, some anisole isomers, and some other components (352). Upon incubation, TCDD became less easily extractable while the other components were in comparison readily extractable, leading to artificial enrichment of the isomer during the analysis. This could cause misinterpretation of experimental results. Formation of metabolites would be expected to increase with time unless precluded by a toxicity threshold or by further metabolism.

Finally, since TCDD is only present in the environment as a contaminant of other chemicals, the analytical procedure must be able to measure TCDD at levels lower than a few parts per million (197). Recovery of ^{14}C-TCDD as measured by combustion from soils receiving 1.78 ppm TCDD were 52% after 1 year from Hagerstown silty clay loam containing 2.5% organic matter, and 67% from Lakeland loamy sand containing 0.9% organic matter (246). At an application rate of 17.8 ppm, 89% was recovered from Hagerstown loam and 73% from Lakeland sand. No metabolites were detected. Little $^{14}CO_2$ was evolved from TCDD-treated soils during 10 weeks' incubation. Extracts of soil treated with 2,7-dichlorodibenzo-*p*-dioxin (DCDD) contained a major metabolite in addition to the parent substrate (246). The metabolite was not identified. About 5% of the added radioactivity in a 0.7 ppm application of DCDD to soils was evolved after 10 weeks. TCDD incubated in lake water for 589 days was not altered (458). However, a lake water and sediment system incubated for the same length of time produced metabolites amounting to 1 to 4% of the original substrate. These products were polar (water soluble) and some were extractable and some were nonextractable in chloroform. The addition of nutrients enhanced formation of metabolites.

SUMMARY

The chlorinated and nonchlorinated dioxins and furans have not yet been shown to be utilizable as a sole carbon source for growth and energy. The parent nonchlorinated dibenzo-*p*-dioxin can be hydroxylated by several bacteria. The hydroxylated products accumulate and are recalcitrant to further oxidation. Some of the mono-, di-, and trichlorinated isomers are also oxidized by some bacteria, although at lower rates. TCDD is also hydroxylated by a few species of bacteria. Not all of the metabolites arising from TCDD metabolism have been identified. The

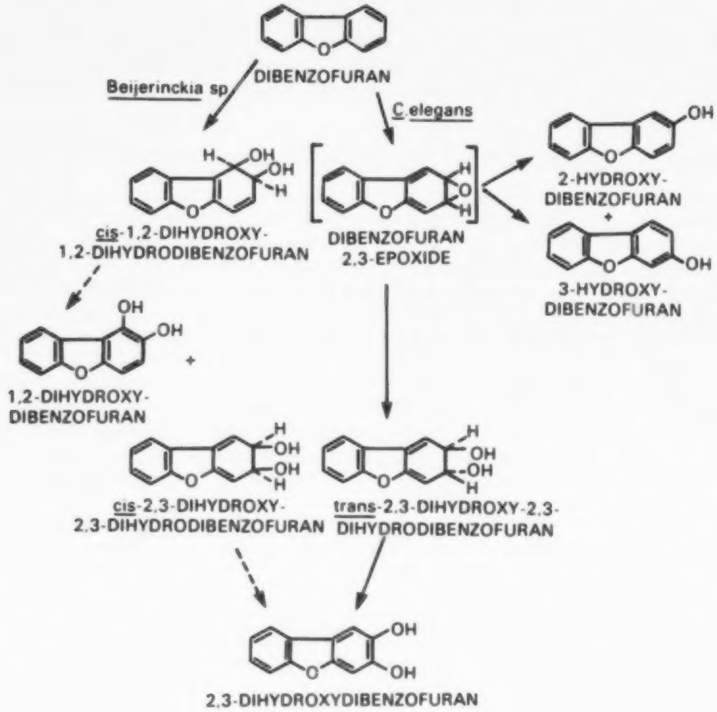

DIBENZOFURAN
Beijerinckia sp.
C. elegans
cis-1,2-DIHYDROXY-1,2-DIHYDRODIBENZOFURAN
DIBENZOFURAN 2,3-EPOXIDE
2-HYDROXY-DIBENZOFURAN
+
3-HYDROXY-DIBENZOFURAN
1,2-DIHYDROXY-DIBENZOFURAN
+
cis-2,3-DIHYDROXY-2,3-DIHYDRODIBENZOFURAN
trans-2,3-DIHYDROXY-2,3-DIHYDRODIBENZOFURAN
2,3-DIHYDROXYDIBENZOFURAN
OH
H
O

Figure 61. Oxidation of dibenzofuran by *Beijerinckia* sp. and *C. elegans*. Bracketed compound is hypothetical intermediate. Dashed lines indicate postulated reactions. Adapted from Reference 77.

dibenzofurans have been less well studied, although it has been demonstrated that the nonchlorinated substrate can be hydroxylated by both bacteria and fungi. Studies of the metabolism of these compounds have been hampered by the difficulty of extraction and product analysis, and by the extreme toxicity of TCDD, the isomer of greatest interest.

SECTION 16

BIODEGRADATION OF CHLORINATED AROMATIC COMPOUNDS IN SCALED-UP BIOLOGICAL WATER RELATED TREATMENT PROCESSES

INTRODUCTION

The previous sections have demonstrated that a wide variety of chlorinated aromatic compounds are subject to biodegradation by a diversity of pure and mixed bacterial cultures. The significance of this information relates to the perceived potential for both environmental and wastewater biodegradation and/or detoxification of chlorinated aromatic pollutants. The extrapolation of such laboratory-derived results to environmental degradation and waste treatment is imprecise due to optimization, acclimation and high cell density cultures employed in most biochemical and physiological studies, which are rarely if ever met in real world biodegradation scenarios. In addition, real world complexity of the environmental matrix in which biodegradation occurs frequently necessitates the use of imperfect measures of biodegradation that cannot readily be correlated with those used to assess biodegradation in a laboratory environment.

Much of the work assessing biodegradation potential has been done in small-scale laboratory glassware with the hope that adequate comparisons might be drawn to full-scale environmental or treatment systems. Many factors may vary between the small-scale lab systems and the full-scale systems. A summary of factors that influence organic biodegradability is presented in Tables 6, 7, and 8 (385). Small-scale lab tests can assess the importance of many of these factors in well-designed, controlled experiments. However, many issues of importance in relating small-scale test results to full-scale process performance depend on interactions of the various individual factors and the rates of material and biomass changes. These often are influenced by the physical design of the system and mass and energy transfer considerations. The turbulence and mixing potential of the system are also of major importance.

For these reasons, environmental scientists and engineers have turned to larger scale experiments to simulate the design and physical features of the full-scale system, whether a treatment or an environmental system. In these systems, material and energy kinetics can be measured, competitive abiotic processes can be studied, and these results can be linked to mathematical models to describe the system and allow scale-up to full-scale systems with more certainty.

This chapter focuses on scale-up studies to determine the biodegradability of chlorinated aromatics reported in the literature. Much information is available on continuous wastewater treatment systems. This was emphasized in this chapter over the environmental microcosm work, since the focus of this work is on treatability as

TABLE 6. CHEMICAL FACTORS INFLUENCING ORGANIC BIODEGRADABILITY IN WATER*

Chemical factors	Consequences
Substrate structural considerations	
Molecular weight or size	Limited active transport
Polymeric nature	Extracellular metabolism required
Aromaticity	Oxygen-requiring enzymes
Halogen substitution	Lack of dehalogenating enzymes
Solubility	Competitive partitioning
Toxicity	Enzyme inhibition, cell damage
Xenobiotic origin	Evolution of new degradative pathways
Environmental factors	
Dissolved oxygen	O_2-sensitive and O_2-requiring enzymes
Temperature	Mesophilic temperature optimum
pH	Narrow pH optimum
Dissolved carbon	Organic/pollutant complexes are concentration dependent for growth
Particulates, surfaces	Sorptive competition for substrate
Light	Photochemical enhancement
Nutrient and trace elements	Limitations on growth and enzyme synthesis

*Reference 385.

TABLE 7. BIOLOGICAL FACTORS INFLUENCING ORGANIC BIODEGRADABILITY IN WATER*

Biological factors	Consequences
Enzyme ubiquity	Low frequency of degradative species
Enzyme specificity	Analogous substrates not metabolized
Plasmid encoded enzymes	Low frequency of degradative species
Enzyme regulation	Repression of catabolic enzyme synthesis required acclimation or induction
Competition	Extinction or low density populations
Habitat selection	Lack of establishment of degradative populations
Population regulation	Low population density of degradative organisms

*Reference 385.

TABLE 8. EXPERIMENTAL FACTORS INFLUENCING ORGANIC BIODEGRADABILITY*

Experimental factors	Problems encountered
Analytical method	
Substrate disappearance	Competing abiotic processes
Biotransformation	Complex analysis
Mineralization	Incomplete biochemical pathways
Scale up/down	Comparability among reactor designs and effects on kinetics
Feedstock complexity	
Chemically/biologically defined	Poor simulation and predictability
Complex waste/wastewaters	Difficult interpretation

*Reference 385.

opposed to persistence in the environment. Little information was found on scale-up studies in the soil matrix.

PENTACHLOROPHENOL

Pentachlorophenol has been studied in several scaled-up systems. These include studies in aerobic "fiber-wall" reactor systems (140) as well as more conventionally-designed lab activated sludge systems. (37, 129, 315.)

A lab-scale test with a continuous, aerobic, "fiber-wall" reactor was used to study the bio-oxidation of PCP in a synthetic and in an authentic wood-preserving wastewater (140). In the synthetic case, the concentration of PCP in the feed was 20 mg/l, the COD was 300 mg/l, the acclimation period was 15 days, and the operational period was 30 days. Reagent grade, commercial grade, and improved commercial grade pentachlorophenol were used. The improved commercial grade had fewer impurities, including chlorodioxins. Table 9 shows a survey of the results of these experiments. Other concentrations were also tested. These data show a general inhibition of the disappearance of PCP in the commercial preparation relative to the reagent and improved commercial grade preparations. Presumably, this is related to impurities present, possibly chlorodioxins. Actual waste rivaled the synthetic tests in PCP degradation performance. No proof of mineralization or estimation of other fate mechanisms was given in these tests.

A series of lab-scale continuous-stirred tank reactors (CSTR) was used to determine the aerobic biodegradation of PCP in wastewater treatment applications (315). The testing protocol included a phase where the inoculum was acclimated to PCP over 90 days from initial concentrations of 1 mg/l to 20 mg/l of PCP in a "fiber-wall" reactor. This sludge was then introduced into continuous-stirred tank reactors with no sludge or cell recycle. The hydraulic residence times (HRT) and the mean cell residence times (MCRT) were, therefore, equal and ranged from 3.2 to 18.3 days. Data collected included disappearance data on COD and PCP as well as reactor suspended solids concentrations. Proof of mineralization of PCP by use of ^{14}C-PCP was employed in related batch studies and fate information was collected on sorption and stripping mechanisms in adjunct batch experiments. No confirmatory

TABLE 9. THE EFFECT OF PENTACHLOROPHENOL PURITY ON DISAPPEARANCE IN CONTINUOUS SYSTEMS*

Parameter	Reagent grade	Commer-cial grade	Improved commer cial grade†	Actual waste
Feed PCP (mg/l)	20	20	20	17.8
Feed COD (mg/l)	515	515	515	1336
Hydraulic residence time (hr)	6	6	6	6
Effluent PCP (mg/l)	0.53-0.7	1.9	0.3	0.2
Effluent COD (mg/l)	15.8-45.6	29.1	52.8	216
Activated sludge initial pentachlorophenate degrading capacity (mg PCP g cell - 1 hr -)	0.4-0.49	0.11	0.4	

*Reference 140.

98% pure PCP.

Blend from four manufacturers, 75-85% pure.

†Same PCP concentration as commercial grade, "substantially reduced" chlorodioxins. Dow Chemical Co., Improved Commercial Grade Penta (XD-8108.00L).

specific analysis of radiolabeled intermediates in the effluent or in the biomass are offered.

Results from this study include a first order kinetic rate constant, (the maximum specific growth rate, μm, divided by the Monod saturation constant, K_s) of 0.00171 $\mu g^{-1} d^{-1}$ with a minimum attainable PCP CSTR reactor concentration of 27 μg/l. Aqueous phase concentrations of PCP ranged between 51-293 μg/l in the reactor. PCP had little effect on the removal of other COD in this study. The batch fate testing indicated that neither stripping nor sorption were significant PCP removal mechanisms and PCP was mineralized with some carbon incorporated into the cellular material.

A synthetic waste containing PCP was treated in a continuous lab-scale activated sludge system consisting of a 6.25-l mixed liquor vessel and a 1.66-l external clarifier (129). Air was added at the rate of 6.25 l/min through a sintered glass sparger. Sludge was wasted at 15 min intervals throughout the experiment. HRTs ranged from 8.9 to 10.4 hours and the MCRT was maintained at 6.2 days. Parameters measured included total solids, sludge volume index, PCP and reducing sugar concentrations in the clarifier, and clarifier effluent turbidity.

Screened wastewater treatment plant sludge was acclimated to PCP using a fill and draw reactor. An *Arthrobacter* sp. strain (ATCC 33790) was also added to another acclimation reactor with the effect of a lag period reduction to 1-3 days as opposed to over 6 days for the unamended sludge.

Steady-state operation of the activated sludge reactor achieved reductions in concentration from 40 mg/l PCP to about 1 mg/l. Unsteady-state transient conditions were studied by increasing the feed to 120 mg/l. Shock loading effects were analyzed with the use of kinetic models. Systems where the *Arthrobacter* sp. was added continuously showed a considerably improved transient response to the shock load than did the acclimated systems without addition of the strain.

Continuous lab-scale activated sludge units, consisting of 11-l aeration factors (air sparged in at ≈ 2 l/min) and 4-l external clarifiers with partial sludge recycle were challenged with 8.6 mg/l of PCP (37). The feed stream consisted of a pulp mill foul condensate with substantial amounts of non-PCP carbon, largely as methanol. Mean cell residence times were 4.9 and 9.3 days and hydraulic residence times were 25.4 and 24.0 hours, respectively. PCP was added as U-^{14}C-PCP and label analysis and specific PCP analysis were performed on the mixed liquor supernate as well as waste biomass and offgas. Other operating conditions are reported as well.

Unlike the other studies, the emphasis of this study was to develop information on PCP sorption on biomass and, therefore, a biomass not acclimated to PCP was used. BOD_5 and TOC removals from the aqueous phase were 63 to 69% and 86 to 96%, while PCP removals were 11.6 to 7.0%, depending on the reactor MCRT. Essentially, all of the PCP removal was either sorbed to or soluble in the aqueous component of the waste sludge. No significant biological transformation or mineralization was evident and stripping of PCP was below the detection limit. Good accountability was found for both labeled and unlabeled PCP.

Batch sorption tests were also incorporated and comparison of the batch data and the continuous runs suggests that the data fits a Langmuir-type isotherm with an apparent saturation of the biomass at an aqueous phase concentration of 2 mg/l PCP. The PCP data collected at concentrations less than the apparent saturation concentration agree well with a proposed sorption equilibrium equation based on the PCP octanol-water partition coefficient. An equation proposed for estimation of stripping based on the Henry's law constant for associated PCP was found to exaggerate the amount stripped. However, use of the Henry's law constant for the pentachlorophenate form was expected to show better agreement. No such Henry's law constant is available.

Consistent with information in earlier chapters, PCP has been found to biotransform in scaled-up systems using acclimated biomass or systems amended with known PCP degraders. No scaled-up studies have attempted to elucidate transformation products other than $^{14}CO_2$, but complete mineralization is strongly indicated in at least one study. Inhibition of other compounds (potentially chlorodioxins) on the biomass has been noted and, therefore, the waste matrix in which the PCP resides may be important in determining the extent and rate of biodegradation. Conventionally-designed activated sludge systems operating with biomass not acclimated to PCP or experiencing transient shock loads either fail to achieve biotransformation of PCP or do so at greatly reduced rates. In these cases, PCP removal from the aqueous phases is poor and sorption to biomass (or other suspended solids) is expected to be a significant removal mechanism.

In summary, effective continous PCP degradation appears to require a biomass with specific PCP degradative capability (PCP degrader sub-population) as evidenced by the importance of acclimation and a steady and transient-free feed. Systems allowing longer cell residence times may also have an advantage over designs incorporating short cell residence time, although evidence here is not conclusive.

CHLORINATED BIPHENYLS

Several studies have occurred in which various mixtures and/or specific congeners of chlorinated biphenyls (PCBs) have been tested for biodegradability in larger, continuous experiments. These have included tests on consortia taken from operating wastewater treatment plants (200, 236, 438), as well as tests using specific organisms (280). Still other research has been completed focusing on the fates of PCBs in environmental microcosms (272).

PCB commercial mixtures (Aroclors 1221, 1016, 1242, 1254, and MCS 1043) were tested for biodegradation using a lab-scale activated sludge test (438). Because of the test protocol, evaluation of the HRT and MCRT was not possible. The sludge inoculum was obtained from a municipal treatment plant and was acclimated for several weeks on a synthetic feed composed of glucose, nutrient broth, and KH_2PO_4. PCBs were not included in the acclimation feedstream. Initial MLSS concentrations were adjusted to 2500 mg/l. PCBs dissolved in ethanol were injected into the reactors. Disappearance of PCBs from the mixed liquor was measured using a hexane extraction followed by specific GC-EC or UV analysis. PCB spikes into the mixed liquor were used to measure analytical recoveries of PCBs from the liquor containing biomass and supernate.

Sorption on biomass was checked by sonic homogenization and extraction of the mixed liquor from an Aroclor 1016 run and extraction in the standard manner. Comparison with results without sonic homogenization showed similar PCB recoveries. No sorption checks were made with higher chlorinated PCBs, nor was proof of mineralization or biotransformation reported. Stripping was checked with Aroclor 1221, MCS 1043, and Aroclor 1016 by use of hexane offgas scrubbers connected to the reactors. Stripping rates of 4.2, 6.1, and 3.6%, respectively, were reported for the above PCB mixtures.

Results of these tests, equating disappearance with degradation, are presented in Table 10. A 48-hour cycle with an addition rate of 1 mg PCB over this interval was used.

TABLE 10. DISAPPEARANCE OF COMMERCIAL CHLOROBIPHENYL MIXTURES*

Mixture	Percent chlorine of mixture	Percent disappearance during test
Biphenyl	0	100
Aroclor 1221	21	81 ± 6
MCS 1043	30	56 ± 16
Aroclor 1016	41	33 ± 14
Aroclor 1242	42	26 ± 16
Aroclor 1254	54	15 ± 38

*Reference 438

A lab study of activated sludge challenged with carbon radiolabeled 2,5,4'-trichlorobiphenyl and 2,4,6,2',4'-pentachlorobiphenyl was performed to determine the fates of these compounds in biological processes (200). Municipal sewage sludge was placed in an aerated glass column and the offgas was scrubbed in hexane and toluene to recover stripped ^{14}C-PCB. No acclimation of the sludge to PCBs was reported. Initial concentrations were 0.178 mg/kg and 0.231 mg/kg trichlorobiphenyl and pentachlorobiphenyl, respectively.

Metabolic byproducts of the trichlorobiphenyl were found in the sludge while none were found in the aqueous phase. One percent of the ^{14}C-trichlorobiphenyl was estimated to undergo biodegradation. The degradation products were found to be less volatile than the parent compound and, therefore, accumulated in the biomass. No evidence of degradation of the pentachlorobiphenyl was found in this study.

A lab-scale semi-continuous test using a "fill-and-draw" technique (236) was performed using municipal sludge acclimated over 3 months on 1,5 and 10 μg/l of Kanechlor 500 (a PCB product with its main component being pentachlorobiphenyl). The units were aerated for 12 hours and then settled for 0.5 hours, after which time the clear supernate and sludge in excess of 25% of the reactor volume was wasted. The reactor was recharged with synthetic feed containing the PCBs, glucose, sodium glutamate, and inorganic nutrients.

The BOD of the feed was 320 mg/l. Specific PCB analysis in the sludge included centrifugation of the sludge, digestion of the solids with methanol-KOH, and subsequent hexane extraction. The aqueous phase was extracted with hexane and extracts were combined, water washed, dried with anhydrous Na_2SO_4, cleaned up with a Florisil column, concentrated, and analyzed by GC-EC. COD measurements and respirometric measurements were also taken during the study.

In batch respirometric tests, oxygen uptake of biomass with 1 and 5 μg/l PCBs was stimulated relative to the control without PCBs. Semicontinuous reactors fed up to 10 μg/l PCBs experienced high BOD removal efficiencies (98.6 to 99.1%). Major removals of PCBs in the semicontinuous reactors were found at all feed concentrations over the 12 hour aeration period. Equilibrium concentrations were achieved during the first hour of the period. Table 11 shows the distribution of PCBs in the semicontinuous reactors for sludge acclimated at 1, 5, and 10 μg/l PCB.

TABLE 11. DISTRIBUTION OF KANECLOR 500 IN ACTIVATED SLUDGE SEMICONTINUOUS SYSTEMS*

Sludge PCB acclimation concentration μg/l	Initial PCB concentration μg/l	Removal in wasted sludge (%)	Remaining in effluent (%)	Unaccounted (%)
1	0.6	45.6	31.3	23.1
5	0.48	75.9	15.0	9.1
10	0.85	81.5	12.1	6.4

*Reference 236.

The majority of the PCBs charged to the system were removed with the wasted sludge. The authors tested stripping of PCBs in their semicontinuous reactors at air flow rates of 0.1 l/min of air per l of mixed liquor and found significant disappearance (65%) after 20 h aeration. They conclude that stripping could account for the PCB losses experienced in the semicontinuous runs. Finally, challenge of a municipal anaerobic digester sludge with 31 μg/l of PCBs (wet weight) incubated at 38°C for 40 days showed no disappearance of the starting PCB material. Resistance to anaerobic biotransformation was concluded.

A lab-scale continuous aerobic study of the degradation of Aroclor 1221 by a *Pseudomonas* sp. strain 7509 culture is reported (280). Inocula were acclimated on a feed in which Aroclor 1221 was the sole carbon source. A fermentation vessel was used as the reactor and raw sewage with a BOD_5 of 140 to 170 mg/l, fortified with 20 mg/l each of nitrogen (as NH_4Cl) and phosphorus (as $KH_2PO_4+K_2HPO_4$), was amended with Aroclor 1221 at concentrations of 50 and 100 mg/l. The waste was fed at rates between 13 and 91 ml/hr to the 14-l reactor in which the agitator rate and the dissolved oxygen concentration was monitored. The HRT ranged from 4.6 to 32 days and was equal to the MCRT.

The broth (or mixed liquor) was sampled periodically and acidified prior to hexane extraction. GC-FID was used for disappearance analysis. No distinction can be made between the compounds in the aqueous and biomass compartments of the mixed liquor since the solids were not separated prior to analysis. Neither stripping nor sorption was measured and no proof of transformation or mineralization was reported.

At high HRTs (16 to 32 days), all of the Aroclor 1221 fed disappeared. At lower HRTs (4.6 to 10.7 days), some of the specific congeners began to appear in the mixed liquor indicating, according to the author, a preference of the organisms for certain congeners. The implication is that under nonstressed conditions, biphenyl, 2-chlorobiphenyl, and 4-chlorobiphenyl will degrade readily while 2,2′-dichlorobiphenyl, 2,4′-dichlorobiphenyl, and 4,4′-dichlorobiphenyl are more recalcitrant. 2,4′-dichlorobiphenyl was found to build up as an indicator of the lower biodegradation rates. Aroclors 1016 and 1254 were also tested with resulting accumulation of all of the components. Switching from continuous to batch operation indicated disappearance of many of those congeners. Specific congeners were not identified in the Aroclor 1016 and 1254 runs.

Fates of 2,2′,4,5′-tetrachloro-, 2,2′,4,4′,5,5′-hexachloro- and 2,2′,3,3′,4,4′,5,6′-octachlorobiphenyls were introduced into a model system that included sediment, water, and air compartments (272). The model systems were fitted with a special gas bubbling device to enable investigation of removal by jet drop entertainment, and were operated in the dark. Anaerobic, sterile (bactericidal $HgCl_2$), and aerobic biotic systems with and without macroinvertebrates were studied. The macroinvertebrates were grown in separate vessels and then added to the model systems. The equivalent of 7500 chironomids/m^2 (*Chironomus plumosus*-type) and 25,000 tubificids/m^2 (*Tubifex tubifex*) were added to the system. Gas flow to the system was estimated to be from 0.00005 to 0.003 ml/min of gas per ml of water. Thus, air flow to liquid volume ratio was from 30 to 2000 times less than that found in diffused air biological treatment systems. ^{14}C-PCB was used and no specific compound analysis was performed on the parent compound or possible metabolites or conversion products. Congeners recovered in each experiment averaged 68%, 40%, and 31% for tetra-, hexa-, and octachlorobiphenyls, respectively. Distribution of PCBs in the various tests are found in Tables 12, 13, and 14.

Although this experiment was intended to simulate a natural ecosystem and clearly differs from experiments on engineered systems, some conclusions are suggested that may relate to engineered treatment systems. First, although the vast majority of the congeners partition with the sediments (90 to 99.9%), the presence of biomass (especially the macroinvertebrates) was a determinative factor in the partitioning related to dispersion of sediment particles and jet-drop entrainment. In an engineered system, the turbulence of the system may override the turbulence from the macroinvertebrates with the result of much greater suspended material potentially being available for adhesion to walls and other surfaces. Of potentially greater importance is increased jet-drop entrainment, because of higher suspended solids concentrations and substantially higher air flow to water volume ratios in the engineered system. Truly aerosolized jet drops may be collected in scrubbers or filters and therefore may be included in measures of stripping potential, even though the substrate is actually not a vapor but a mechanically-carried liquid-solid droplet.

Unfortunately, no literature addressing this potentially important removal mechanism in engineered systems is evident.

TABLE 12. DISTRIBUTION OF 2,2′,4,5′-TETRACHLOROBIPHENYL IN A SEDIMENT-WATER-AIR MODEL SYSTEM*

Compartment	Aerobic with macro-invertebrates	Aerobic without macro-invertebrates	Sterile	An-aerobic
Sediment	97.7†	98.3†	99.8†	99.3†
Dissolved in water	0.03	0.06	0.02	0.03
Particles in water	0.06	0.03		
Macroinvertebrates	0.55			
Glass walls (particle adhesion)	0.20			0.02
Glass walls (extractable)	0.10	0.02		
Air filters	0.03	0.01		0.02
Vessel stoppers	0.06	0.08		0.04
Surface microlayers	0.03	0.01		
Jet-drop impactors	0.93	1.48	0.18	0.04

*Reference 272.

†All values as percent of recovered compound.

TABLE 13. DISTRIBUTION OF 2,2′,4,4′,5,5′-HEXACHLOROBIPHENYL IN A SEDIMENT-WATER-AIR MODEL SYSTEM*

Compartment	Aerobic with macro-invertebrates	Aerobic without macro-invertebrates	Sterile	An-aerobic
Sediment	96.1†	99.9†	98.9†	99.9†
Dissolved in water	0.06			0.02
Particles in water	0.14			
Macroinvertebrates	2.73			
Glass walls (particle adhesion)	0.48	0.01		0.02
Glass walls (extractable)	0.02		0.17	0.02
Air filters	0.01		0.02	
Vessel stoppers			0.02	
Surface microlayers	0.04	0.03		0.03
Jet-drop impactors	0.36	0.03	0.10	0.03

*Reference 272.

†All values as percent of recovered compound.

TABLE 14. DISTRIBUTION OF 2,2′,3,3′,4,4′,5,6′-OCTACHLOROBIPHENYL IN A SEDIMENT-WATER-AIR MODEL SYSTEM*

Compartment	Aerobic with macro-invertebrates	Aerobic without macro-invertebrates	Sterile	Anaerobic
Sediment	90.0†	99.6†	99.8†	99.4†
Dissolved in water	0.10	0.03	0.06	0.45
Particles in water	1.00	0.02	0.09	0.01
Macroinvertebrates	3.00			
Glass walls (particle adhesion)	4.67	0.0		
Glass walls (extractable)	0.07			0.01
Air filters	0.01			
Vessel stoppers	0.30			0.05
Surface microlayers	0.07	0.03		0.11
Jet-drop impactors	0.99	0.24		0.04

*Reference 272.
†All values as percent of recovered compound.

A single scaled-up study has been reported to date that conclusively demonstrates PCB biodegradation. In this study, metabolic byproducts accounting for about 1% of the trichlorobiphenyl were recovered. No other studies present conclusive evidence for biodegradation. This is largely due to use of parent compound disappearance data for analysis and lack of proof for biotransformation or mineralization. No work is evident using labeled congeners to support an argument for enzymatic processes. Several studies have shown major potential for PCB disappearance related to stripping and to sorption on biomass or other solids (sediments). Analytical difficulty in extractive recovery of the higher chlorinated congeners from sediments suggests that in the absence of methods to digest the biomass or to determine specific recoveries of the higher congeners in the biomass, extractive analysis of the biomass or the total mixed liquor (biomass and supernate) may not recover substantial portions of the parent compounds strongly sorbed to the cellular or solids matrix. Spiking with a PCB mixture and complete extractive recovery is not conclusive proof of recovery of PCBs from unknown biomass samples unless no saturation effect of the biomass with sorbed PCBs exists and sufficient time is allowed for the PCBs to achieve equilibrium partitioning with the biomass. Absence of either condition would lead to more of the spiked sample residing in the aqueous compartment and misleadingly high apparent recovery values. In other words, special caution must be taken when equating PCB-spiked mixed liquor or biomass recovery with actual sample PCB recovery.

The strongest evidence of biodegradation, notwithstanding the above comments, is the study by Liu (280) in which ratios of the specific congeners in Aroclor 1221 change depending on the operating conditions. It is difficult to see how sorption or stripping mechanism would vary as widely as reported for the same congeners under different operating conditions unless enzymatic processes are at work. Also the PCB mixture studied is comprised of congeners that, in other microbial tests (reported in an earlier chapter), have been shown to undergo biotransformation.

In general, further scaled-up studies on PCBs are needed that include analytical protocols to offer proof of biotransformation or mineralization, quantify the sorption and stripping mechanisms, and study other potentially important mechanisms such as jet-drop entrainment in engineered systems, before engineered biological processes may be considered for PCB treatment.

DICHLOROPHENOL

A lab-scale activated sludge reactor with sludge recycle (liquid volume of aeration tank, 3 l) was used to study the simultaneous biodegradation of 2,4-dichlorophenol (DCP) and phenol (33). The phenol concentrations ranged from 14.9 to 45.7 mg/l and the DCP concentrations ranged from 52.4 to 121 mg/l. A 1:1 carbon ratio of both substrates was desired. The sludge was previously acclimated to phenol and then was acclimated to DCP by gradual replacement of the phenol in the feed with DCP. The acclimation process required 70 days to complete. The reactors were operated with HRT between 2.5 to 6.25 hr and MCRT of between 1.75 and 10.7 days.

MLVSS concentrations ranged between 46 and 299 mg/l. Analysis focused on substrate disappearance with no determination of stripping or sorption. Chloride analysis as a test of mineralization and COD determinations did not account for the phenol or DCP disappearance, implying biotransformation of the substrates.

Biokinetic rate constants for the runs yielded disappearance rate constants of 0.00098 l $mg^{-1}hr^{-1}$ for phenol and 0.045 hr^{-1} for DCP. The Monod half-saturation constant for DCP was 63 mg/l. Yield coefficients for phenol and DCP were 0.67 mg VSS/mg phenol and 0.39 mg VSS/mg DCP, respectively. A combined biomass decay coefficient of 0.014 hr^{-1} was presented.

A mathematical rationale leading to deterministic estimates of fates from biological waste water treatment processes has been proposed (38). For continuous, complete-mix, activated sludge units where the biological disappearance of the parent compound is described by a rate equation first order in substrate concentration, and where sorption is occurring at concentrations below any potential biomass saturation concentration, the following equations are proposed for first estimates of the percent substrate stripped, sorbed, and wasted in the waste biomass, and biotransformed to another compound:

$$\mathrm{REM}_s = \frac{A}{1 + A + S + B} \tag{2}$$

$$\mathrm{REM}_u = \frac{S}{1 + A + S + B} \tag{3}$$

$$\mathrm{REM}_b = \frac{B}{1 + A + S + B} \tag{4}$$

$$\mathrm{REM}_e = \frac{1}{1 + A + S + B} \tag{5}$$

$$A = \mathrm{HRT}\,\frac{X\ K_{ow}\ f_L}{1000\ \rho_L\ \mathrm{MCRT}} \tag{6}$$

$$S = \mathrm{HRT}\ K^{st} = \frac{Q_{air}}{V}\,3.71 \times 10^{-3}\ (H_c)^{1.045} \tag{7}$$

$$B = \mathrm{HRT}\ K^{b} \tag{8}$$

where: REM_s, REM_{st}, REM_b, REM_e are the percent removals of the substrate from the system by the sorption, stripping, biological transformation, and effluent fate mechanisms, respectively,

HRT is the hydraulic residence time of the activated sludge system (hr),

X is the concentration of biomass as MLSS (mg/l),

K_{ow} is the substrate octanol-water partition coefficient (concentration in octanol/concentration in water),

f_L is the fraction of lipids or lipophilic compounds in the biomass (weight fraction),

ρ_L is the mean density of the lipophilic biomass compounds (g/l),

MCRT is the mean cell residence time for the biomass in the systems (hr),

Q_{air}/V is the ratio of the air flow rate into the system to the system hydraulic volume (min^{-1}),

K^b is a biological disappearance rate constant, first order in substrate concentration (hr^{-1}).

Equations 6 to 8 are discussed individually in separate papers (37, 385, 437).

If assumptions are made such that the equations described above are applicable to the DCP experiment discussed earlier, (complete-mix system, $Q_{air}/V = 0.1\ \mathrm{min}^{-1}$, H_c of DCP = 13.4 torr L mol^{-1}, K_{ow} of DCP = 1202, etc.) then the data reported can be used to calculate K^b for each experimental series and the mechanism removals can be estimated. Table 15 presents the results of this analysis. It may be concluded from this analysis that stripping can be a significant removal mechanism, especially in the instance where K^b is relatively slow. In run 4, 16% of the DCP removed from the system (other than in the effluent) was stripped. As the biotransformation rate increased, the stripping potential was vastly reduced to less than 0.1% of the total DCP removed (other than in the effluent) (Run 1). Sorption of DCP to the waste sludge taken from the system is not a significant fate mechanism for DCP. However, depending upon the level of extracellular water wasted with the biomass (waste sludge solids concentration), more DCP could be lost in the waste sludge than that shown in Table 15.

TABLE 15. FATE ESTIMATES OF 2,4-DICHLOROPHENOL REMOVAL FROM A LAB ACTIVATED SLUDGE SYSTEM USING PROPOSED EQUATIONS[*]

				Percent removal of DCP by			
Run	MCRT (hr)	HRT (hr)	Calculated K^b (hr^{-1})	Sorption	Stripping	Biotransformation	Effluent
1[+]	257	25	0.075	0.008	0.034	63.0	33.6
2[+]	42	6.25	0.043	0.67	2.0	21.6	76.8
3[†]	109	6.25	0.070	0.25	1.8	29.8	68.1
4[†]	20	6.25	0.020	0.78	2.2	10.8	86.2

[*] Data taken from Reference 33.

[+] Runs with phenol and 2,4-dichlorophenol at a ratio of 1 to 1 carbon from each substrate.

[†] Runs with glucose and 2,4-dichlorophenol at a ratio of 1 to 1 carbon from each substrate.

Finally, the first order biotransformation rate constants, K^b, are similar to values reported by Beltrame, et al. (33) derived using more conventional empirical biokinetic rate constant methods ($K^b = 0.045 \pm 0.005^{-1}$). Calculated rate constants from Table 10 show wider variance and indicate strong relationships between biological rates and the MCRT.

TRICHLOROCARBANILIDE

Trichlorocarbanilide (TCC) was studied in both lab-scale batch flask tests and continuous activated sludge systems (184a). TCC with ^{14}C label on the 4-chloroaniline (p-chloroaniline or PCA) ring as well as the dichloroaniline (DCA) ring was used.

Sludge was obtained from a municipal wastewater treatment plant. In the activated sludge units, the biomass concentration and air flow rates were controlled at 4000 μg/l as MLSS and 0.05 standard ft^{3}hr of CO_2-free air. Offgas was trapped in an amine solution to recover $^{14}CO_2$. The ^{14}C content of the sludge was determined by a combustion-$^{14}CO_2$ recovery method. 200 μg/l of TCC were added to the feed stream.

Batch flask tests showed that 90% of the theoretical $^{14}CO_2$ evolved from incubation with both raw sewage and activated sludge after 12 weeks. A lag of 2 weeks was noted in the raw sewage before $^{14}CO_2$ evolution. Concentrations of 200 μg/l of TCC resulted in nearly 100% theoretical $^{14}CO_2$ evolution at 12 weeks while concentrations of both 20 μg/l and 2000 μg/l TCC resulted in less mineralization at this incubation time.

Sorption of TCC to activated sludge was determined by contacting activated sludge at concentrations from 0 to 2000 μg/l with 20 to 200 μg/l of TCC. Sorption was calculated based on the disappearance from the aqueous phase over 2 hr. A Freundlich isotherm was generated showing TCC-on-sludge concentrations of 2 and 0.1 μg/l at aqueous concentrations of 78 and 8 μg/l, respectively. Continuous operation of the activated sludge unit at a 10 hr HRT for 90 days showed 20 to 30% of theoretical $^{14}CO_2$ evolution for the dichloroaniline ring while from 40 to 60% of

TABLE 16. FATE OF TRICHLOROCARBANILIDE IN LAB-SCALE ACTIVATED SLUDGE SYSTEMS*

Substrate	Stream	% of Feed	% Recovered
^{14}C-PCA-TCC†	Effluent	3.2	93.4
	$^{14}CO_2$	56.1	
	Activated sludge	34.1	
^{14}C-DCA-TCC§	Effluent	30.3	91.4
	$^{14}CO_2$	25.9	
	Activated sludge	35.2	

*Reference 184a.

†Trichlorocarbanilide with a labeled 4-Chloroaniline ring.

§Trichlorocarbanilide with a labeled dichloroaniline ring.

theoretical $^{14}CO_2$ was evolved from the chloroaniline ring. Table 16 highlights the fate of the radioactivity in the continuous tests.

Although no specific analysis of the sludge was undertaken to determine the chemical composition of ^{14}C found in the sludge (i.e., parent compound, metabolites, cellular material), such analysis was done on the effluent. Chloroaniline, dichloroaniline, aniline condensation products, and unknowns were found in addition to the TCC parent compound.

This study convincingly supports conclusions related to the biotransformation and mineralization of TCC. These findings are consistent with those stated in an earlier chapter on chloroaniline herbicides even though this molecule's structure varies somewhat. In addition, this study is among the earliest work found that considered major fate mechanisms and offers proof of biotransformation or mineralization in a scaled-up biological wastewater system treating chloroaromatic compounds. Unfortunately, lack of information on the MCRT limits the calculation of biokinetic rate constants and the direct extrapolation of these results to other design configurations.

DICHLOROBENZENE

A pilot scale activated sludge system was operated on a side-stream of sewage from the city of Zurich, Switzerland (303). 1,4-Dichlorobenzene was present at all times in the system feed and it was used as an indicator compound to determine the nonbiological removal mechanisms of stripping and sorption on the biomass. The major assumption made in this study was that 1,4-dichlorobenzene was conserved and was not biotransformed at the operating conditions of the study. The aeration vessel volume was 11.3 to 15m^3 and the HRTs ranged from 2.5 to 6.5 hr with MCRTs ranging from 74 to 182 hr. Sorption and stripping mechanisms were quantified but proof of biotransformation (or the absence of) was not provided.

The fates of DCB were reported to be 72% stripped, less than 3% sorbed on wasted sludge, 10% in the effluent, and 15% unaccounted. Data presented in an earlier section indicated that chlorobenzenes (except hexachlorobenzene) can be mineralized but there is a lack of knowledge on the biochemical pathways. The DCB not accounted for in this study may be undergoing biotransformation but the variance in the material balance related to analytical shortcomings precludes a definite statement. A study discussed later in this chapter offers evidence of DCB biotransformation.

COMBINED STUDIES ON SEVERAL CLASSES OF CHLOROAROMATICS

Lab and Pilot Studies

Several studies have occurred using a given experimental protocol on a variety of chloroaromatic compounds. Table 17 shows the classes of chloroaromatics studied for each of the these compounds.

Benzoic acid, 2-chlorobenzoic acid, 3-chlorobenzoic acid, 4-chlorobenzoic acid, 2,4-dichlorobenzoic acid, 2,5-dichlorobenzoic acid, 2,6-dichlorobenzoic acid, 3,5-dichlorobenzoic acid, phenoxyacetic acid, and 2,4-dichlorophenoxyacetic acid were studied in lab-scale continuous flow reactors resembling chemostats (391). The HRT and MCRT were equal in these reactors. The reactors were completely mixed by aeration and used suspended biomass. The lag for acclimation of municipal biomass to the specific substrates was determined. Kinetic disappearance data were collected on the specific substrates and on dissolved organic carbon (DOC) as well. Batch testing also was performed to determine kinetic rate constants for comparison with the continuous tests. Proof of mineralization of the specific substrates was determined by measurement of chloride ion release.

The lag for acclimation of the initial sludge for the monochlorobenzoic acids was in the range of 10 to 20 days. The biomass began to show acclimation to 3,5-dichlorobenzoic acid at about 20 days but the acclimation process was continued through 100 days. 2,5-dichlorobenzoic acid became acclimated abruptly at 100 days. 2,4- and 2,6-dichlorobenzoic acids did not show acclimation during this testing protocol.

The author argues that long term acclimation for some of the compounds is evidence for genetic changes in the organisms as opposed to enzyme induction or population effects. Maximum specific growth rates, μ_m, the Monod half-saturation constant, K_s, and the yield coefficient, Y, are shown in Table 18.

Good agreement was found between μ_m and K_s for the continuous flow reactors and associated batch tests. The MCRT of the systems were found to be strongly related to to the effluent concentrations of the specific substrates. MCRTs of 3 to 15 days were required to achieve a 0.5 mg/l effluent concentration of the monochlorobenzoic acids while 6 to 50 days were needed to achieve 0.25 mg/l effluent concentrations.

TABLE 17. CLASSES OF CHLOROAROMATICS STUDIED IN SEVERAL EXPERIMENTAL STUDIES

	Reference					
Compound	61	127	250	251	391	416
Pentachlorophenol	X		X	X		X
Chlorinated Biphenyls						
Aroclor 1242	X					
Aroclor 1254	X					

(continued)

TABLE 17. (continued)

Chlorophenols						
2-Chlorophenol	X					
2,4-Dichlorophenol	X	X	X	X		X
2,4,6-Trichlorophenol	X					
Chlorobenzenes						
Chlorobenzene	X					
1,2-Dichlorobenzene	X	X	X	X		X
1,3-Dichlorobenzene	X	X				
1,4-Dichlorobenzene	X	X				
1,2,4-Trichlorobenzene	X	X				
Hexachlorobenzene	X					
Chlorobenzoic acids						
2-,3- and 4-chlorobenzoic acid					X	
2,4-, 2,5-, 2,6-, and 3,5-Dichlorobenzoic acid					X	
Chlorophenoxy compounds						
2,4-Dichlorophenoxyacetic acid					X	

Benzoic acid, 2-chlorobenzoic acid, 3-chlorobenzoic acid, 4-chlorobenzoic acid, 2,4-dichlorobenzoic acid, 2,5-dichlorobenzoic acid, 2,6-dichlorobenzoic acid, 3,5-dichlorobenzoic acid, phenoxyacetic acid, and 2,4-dichlorophenoxyacetic acid were studied in lab-scale continuous flow reactors resembling chemostats (391). The HRT and MCRT were equal in these reactors. The reactors were completely mixed by aeration and used suspended biomass. The lag for acclimation of municipal biomass to the specific substrates was determined. Kinetic disappearance data were collected on the specific substrates and on dissolved organic carbon (DOC) as well. Batch testing also was performed to determine kinetic rate constants for comparison with the continuous tests. Proof of mineralization of the specific substrates was determined by measurement of chloride ion release.

The substrates were mineralized to CO_2 and cellular material but no fate measurements were made on stripping or sorption mechanisms. Performance of systems with glucose and the 2,4-dichlorophenoxyacetic acid indicated no effect of glucose on 2,4-D disappearance but lower glucose disappearance rates related to 2,4-D presence. However, no strong inhibition or toxic effects of the substrates on the biomass were seen at feed concentrations of 50 to 200 mg/l. The effluent concentration is, of course, much less than the feed concentrations.

A major study on a variety of organics was undertaken (250, 251, 416). Continuous lab-scale activated sludge units were challenged with chlorophenols, chlorobenzenes, and pentachlorophenol as well as a number of other organics. These compounds were added to a synthetic "base mix" containing ethylene glycol, ethyl alcohol, acetic acid, glutamic acid, glucose, phenol, and various inorganic nutrients. The chloroaromatics were added such that the BOD_5 achieved was $\approx$ 250 mg/l. HRT was held to about 8 hours and the MCRT ranged between 43 and 146 hrs. Stripping was measured by trapping the organics from the offgas on a solid sorbent and all specific compound analysis generally followed EPA analytical protocols. Methodology for

TABLE 18. BIOKINETIC RESULTS FOR 2,4-D AND CHLOROBENZOIC ACIDS*

Compound	μ_m** (day^{-1})	K_s† (mg/l)	Y†† (mg/mg)
2,4-Dichlorophenoxyacetic acid	2.3	5.4	0.14
2-Chlorobenzoic acid	1.0	2.4	0.22
3-Chlorobenzoic acid	0.6	2.0	0.14
4-Chlorobenzoic acid	1.2	1.1	0.25
2,5-Dichlorobenzoic acid	0.6	1.5	0.16
3,5-Dichlorobenzoic acid	0.05	25.3	

*Reference 391.
**Maximum specific growth rate.
†Monod half-saturation constant.
††Yield coefficient.

TABLE 19. FATE OF SEVERAL CHLOROAROMATICS IN A LAB-SCALE ACTIVATED SLUDGE SYSTEM*

Compound	Percent removed by		
	Stripping	Sorption	Biotransformation
2,4-Dichlorophenol			95.2
Pentachlorophenol		0.58	97.3
1,2-Dichlorobenzene	21.7		78.2

*Reference 250, 251, 416.

sorption quantitation was not clear. No proof of mineralization or biotransformation was offered. Acclimation to the compounds was allowed for 4 weeks before 2 months of continuous data collection. The reactor volume was 3 l for aeration and 3.3 l for an internal clarifier. Air flow to the reactors ranged from 2 to 3 l/min. Table 19 presents fate data on specific chloroaromatics. These studies utilized an air flow-to-liquid volume ratio greater than 1 min^{-1} and the resultant data probably exaggerates the stripping mechanism. Disappearance attributed to biotransformation for DCP and PCP are in agreement with earlier studies reviewed in this chapter. Biotransformation of 1,2-dichlorobenzene is in conflict with a study discussed earlier but is consistent with general predictions on the biodegradability of chlorobenzenes.

Full-Scale Studies

Many full-scale biodegradation studies on chloroaromatics are reported. However, only a few have attempted to quantify the abiotic fates of the compounds. Also, variation in the waste composition and flow and in other physical variables often makes it difficult to interpret the results. Two of the most significant examples of full-scale plant studies are discussed here.

A study to determine the fates of priority pollutants for 50 publicly owned wastewater treatment plants has generated input-output data on several chlorinated aromatics including 2-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, pentachlorophenol, chlorobenzene, 1,2-dichlorobenzene, 1,3-dichlorobenzene, 1,4-dichlorobenzene, 1,2,4-trichlorobenzene, hexachlorobenzene, Aroclor 1242, and Aroclor 1254 (61). This work was designed to allow statistical analysis on the occurrence and fates of priority pollutants in the system feedstream, intermediate process streams, system effluent, and waste sludge streams. The material that disappeared in the process was reported but since no air sampling was undertaken, no quantitative attempt was made to distinguish between stripping and biotransformation removal mechanisms. EPA analytical protocols were used and often the compounds were grouped into the "volatile", "acid extractable", and "base neutral" groupings arising from the analytical workup. Sampling periods were for approximately 6 days with 24 hr composite samples, and compounds were often at concentrations near the analytical detection limit. Since the influent was often variable and the sampling period was of the same order of magnitude as the plant's MCRT, material balance data and conclusions must be viewed with caution.

A related study was performed on a single publicly-owned wastewater treatment plant for a 30 day period (127). Over this period, variations in influent flow and substrate concentrations could be more precisely quantified and conclusions regarding compound fate could be made. Table 20 summarizes the removal data of 2,4-dichlorophenol, 1,3-dichlorobenzene, 1,4-dichlorobenzene, and 1,2,4-trichlorobenzene found in this study. The removal calculation sums the substrate entering the system from all streams and the substrate leaving in all streams. Thus, sorption and effluent removal mechanisms are not considered "removed" whereas biotransformation, stripping and other abiotic mechanisms, excluding sorption, are considered "removed".

TABLE 20. MASS REMOVAL OF CHLOROAROMATIC COMPOUNDS IN A FULL-SCALE WASTEWATER TREATMENT PLANT*

Compound	Percent removal in†		
	Primary treatment	Secondary treatment§	Overall treatment
2,4-Dichlorophenol	2	46	47
1,3-Dichlorobenzene	14	30	40
1,4-Dichlorobenzene	0	88	88
1,2,4-Trichlorobenzene	12	79	82

*Reference 127.

† Calculation includes (total mass accounted for in minus total mass accounted for out)/total mass accounted for in. Thus, compound in the aqueous effluent is combined with that found in the waste solids. Removal mechanisms here are biotransformation, stripping, and other abiotic mechanisms excluding sorption.

§Based on activated sludge units alone.

Full-scale plant data requires special planning and careful implementation to yield satisfactory data on biotic and abiotic removal mechanisms. Conclusive material balances often are impeded because of feed variability and low compound concentrations. Use of labeled compounds is expensive at large scale and questions related to the environmental release of labeled compounds exist. Therefore, the capability of collecting data leading to proof of biotransformation or mineralization is limited.

SUMMARY

In order to conclusively establish biodegradation of chlorinated aromatic compounds in larger scale systems and to collect data that are of use in extrapolation and system design, several factors must be included in the experimental design. These include: (1) measurement or prediction of abiotic fate processes, (2) proof of biotransformation or mineralization, and (3) suitable measurement and reporting of important process variables relating to the calculation of biokinetic rates. Failure to include these factors leads to inconclusive results on compound fates or the inability to use the data for predictions on other (even similarly designed) systems.

Calculation of biokinetic rate constants based only on "removal" leads to wide variances in the rate constants for compounds with major abiotic fate tendencies and precludes reliable scale-up and more direct comparison between systems. Only a few scale-up studies are available on chlorinated aromatics in general, and only a subset of these contain data suitable for drawing conclusions relative to biodegradation and which allow comparison between systems. At times, coupling the results from several studies may allow an enlightened judgment regarding biodegradation, but there is no substitute for a single well-designed study. In general, much additional work is needed to generate reliable scale-up data for biological treatment of chloroaromatic compounds.

SECTION 17

OVERVIEW OF MICROBIOLOGICAL DECOMPOSITION OF CHLORINATED AROMATIC COMPOUNDS

Most of the studies reviewed here have explored the metabolism of a single compound by a single organism. A few have reported the metabolites formed in soils or by contrived microbial consortia. Taken together, however, these studies indicate the potential ultimate fate of the chloroaromatic compounds.

The chlorophenoxy herbicides and the chlorobenzenes can be metabolized to chlorophenols (Figure 62). Chlorophenols may in turn be metabolized to chloroanisoles, but the most common route of biodegradation is to chlorocatechols. Phenylamide herbicides and other compounds with nitrogen-containing substituents are metabolized to chloroanilines. The chloroanilines form a variety of products including chlorocatechols (Figure 63). Other products represent alterations of the aliphatic moiety.

Chlorocatechols, in turn, may be metabolized by several different mechanisms to nonchlorinated ring cleavage products (Figure 64). There are two main pathways. One results from *meta* cleavage to form a chlorohydroxymuconic semialdehyde which, after loss of the chloride, forms pyruvate and an aldehyde. The second pathway involves *ortho* cleavage to form β-ketoadipic acid via chloromuconate. The products of this pathway that are incorporated into cell constituents are succinate and acetyl-CoA.

Chlorobenzoic acids may be metabolized by 3 routes (Figure 64), the first through protocatechuic acid (a substituted catechol) to 3-ketoadipic acid. The second route metabolizes chlorobenzoic acids through chlorosalicylic acid to maleylpyruvic acid. Chloronaphthalenes are also metabolized through chlorosalicylic acid. Anaerobic metabolism of chlorobenzoic acids involves reductive dechlorination to benzoate followed by formation of CH_4 and CO_2. The ultimate products of each of these metabolic pathways are either available for incorporation into cellular material or represent ultimate mineralization.

These pathways all represent a general biochemical potential. Whether or not a specific compound is actually metabolized to nonchlorinated products depends on many factors. The compounds which are most readily metabolized are the lower chlorinated forms. However, some compounds with only one chlorine, for instance 2-chloroaniline, are not readily metabolized. Thus both the position and the number of substituents are important in determining the biodegradability of a molecule. It is not only the number of chlorines but also the general form of the molecule itself which governs biodegradability. Compounds such as chlorodioxins and DDT are not shown on these figures because they appear to be highly resistant to substantive microbial attack. The exact physicochemical features of any given molecule that govern its biodegradability remain to be elucidated.

Even chlorinated molecules for which complete pathways of mineralization have been developed are not metabolized in all systems. Although a few compounds are amenable to anaerobic biodegradation, most require the availability of molecular oxygen for ring cleavage. Other environmental parameters which may place

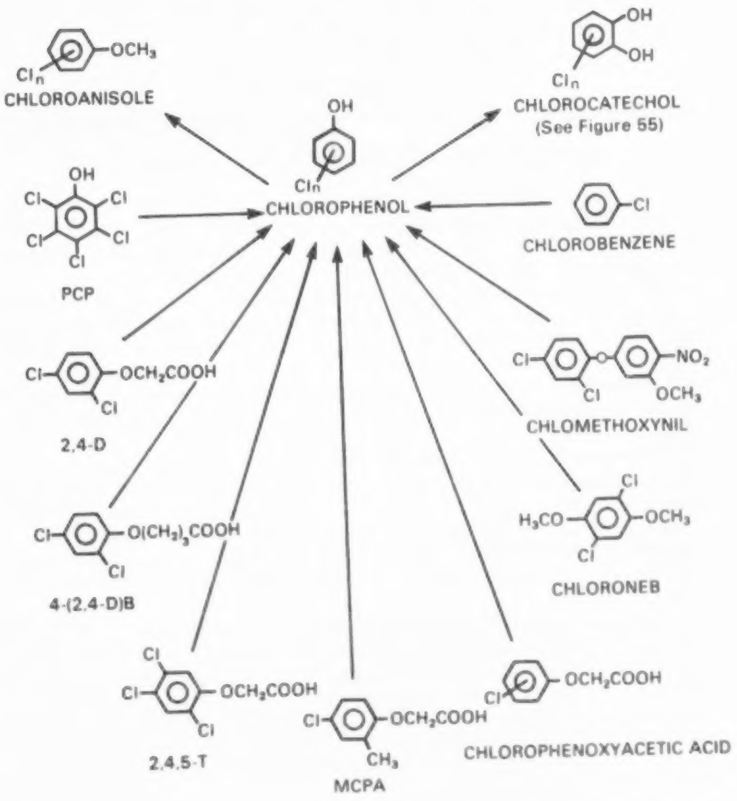

CHLOROANISOLE
OH
CHLOROPHENOL
CHLOROCATECHOL
(See Figure 55)
PCP
CHLOROBENZENE
2,4-D
CHLOMETHOXYNIL
4-(2,4-D)B
CHLORONEB
2,4,5-T
MCPA
CHLOROPHENOXYACETIC ACID

Figure 62. Chlorinated aromatic compounds metabolized to chlorophenols. This figure presents possible pathways extrapolated from various studies. In actual environmental systems a given transformation may be inhibited by a number of factors. Terminal compounds shown may be recalcitrant or insufficient research may exist on which to base a conclusion. Refer to text for further discussion.

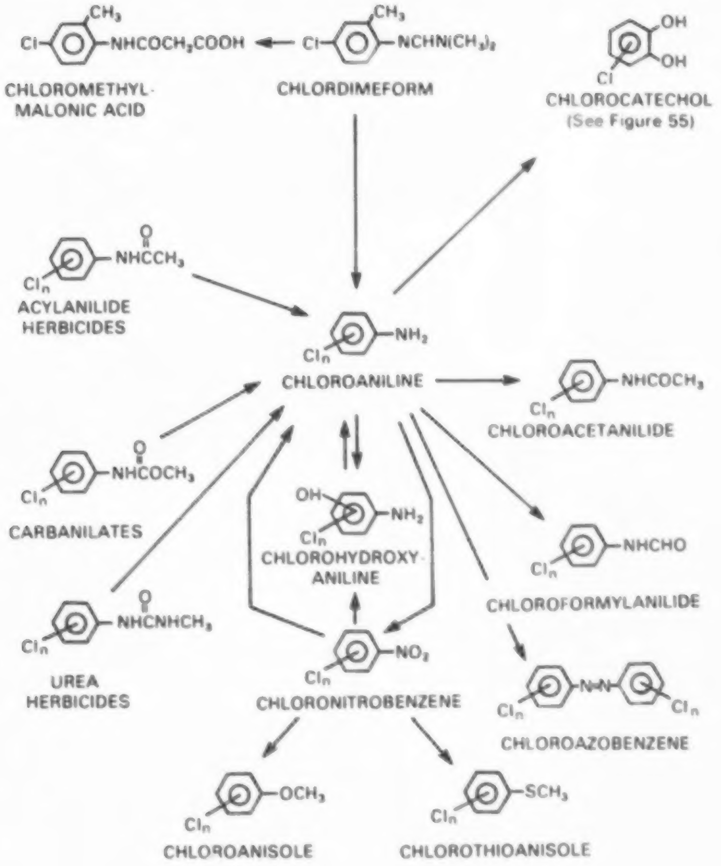

CH₃
Cl–NHCOCH₂COOH
CHLOROMETHYL-MALONIC ACID
CH₃
Cl–NCHN(CH₃)₂
CHLORDIMEFORM
OH
OH
Cl
CHLOROCATECHOL
(See Figure 55)
O
NHCCH₃
Clₙ
ACYLANILIDE HERBICIDES
NH₂
Clₙ
CHLOROANILINE
NHCOCH₃
Clₙ
CHLOROACETANILIDE
O
NHCOCH₃
Clₙ
CARBANILATES
OH
NH₂
Clₙ
CHLOROHYDROXY-ANILINE
NHCHO
Clₙ
CHLOROFORMYLANILIDE
O
NHCNHCH₃
Clₙ
UREA HERBICIDES
NO₂
Clₙ
CHLORONITROBENZENE
N=N
Clₙ
Clₙ
CHLOROAZOBENZENE
OCH₃
Clₙ
CHLOROANISOLE
SCH₃
Clₙ
CHLOROTHIOANISOLE

Figure 63. Chlorinated aromatic compounds metabolized to chloroanilines. This figure presents possible pathways extrapolated from various studies. In actual environmental systems a given transformation may be inhibited by a number of factors. Terminal compounds shown may be recalcitrant or insufficient research may exist on which to base a conclusion. Refer to text for further discussion.

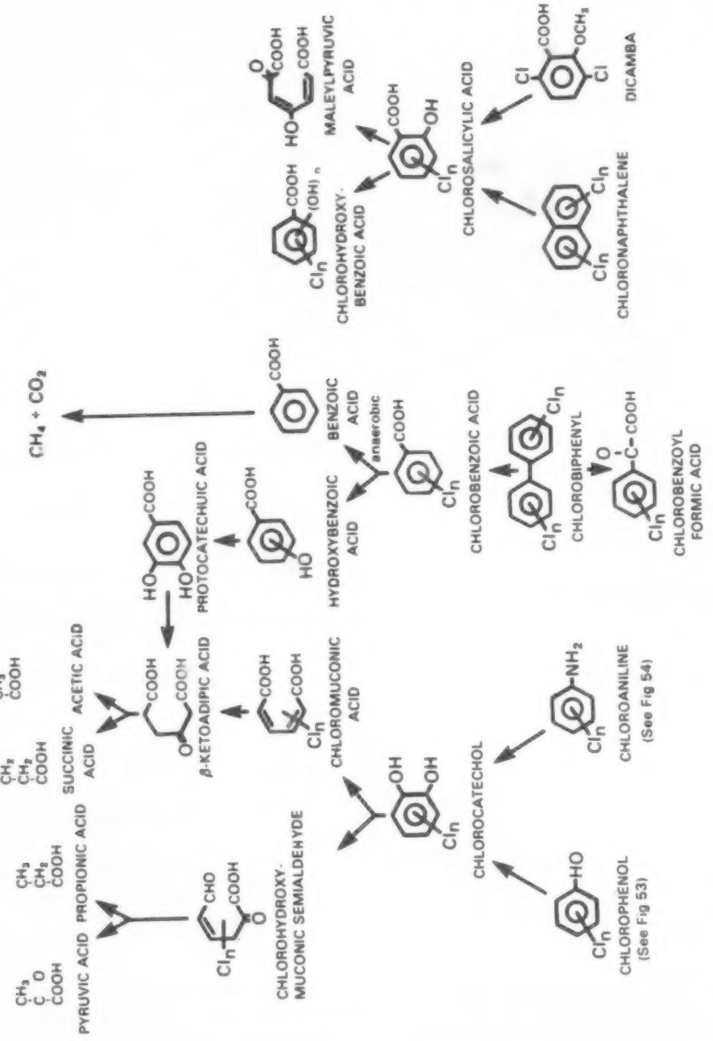

CH₄ + CO₂
PYRUVIC ACID
PROPIONIC ACID
SUCCINIC ACID
ACETIC ACID
β-KETOADIPIC ACID
CHLOROHYDROXY-MUCONIC SEMIALDEHYDE
CHLOROMUCONIC ACID
PROTOCATECHUIC ACID
HYDROXYBENZOIC ACID
BENZOIC ACID
anaerobic
CHLOROCATECHOL
CHLOROBENZOIC ACID
CHLOROBIPHENYL
CHLOROBENZOYL FORMIC ACID
CHLOROPHENOL (See Fig 53)
CHLOROANILINE (See Fig 54)
CHLOROHYDROXY-BENZOIC ACID
MALEYLPYRUVIC ACID
CHLOROSALICYLIC ACID
CHLORONAPHTHALENE
DICAMBA

Figure 64. Metabolism of chlorocatechols, chlorobenzoic acids, and chlorosalicylic acids. This figure presents possible pathways extrapolated from various studies. In actual environmental systems a given transformation may be inhibited by a number of factors. Terminal compounds shown may be recalcitrant or insufficient research may exist on which to base a conclusion. Refer to text for further discussion.

restrictions on biological activity include pH, temperature, and moisture. Upon exposure to the environment, the chemical state of the substrate may be altered to a form resistant to microbial attack.

The chemical itself may be degradable but the system may lack other nutrients necessary for microbial activity. Alternatively, other chemicals present may be preferred substrates, preventing metabolism of the substrate of interest. Another compound may also act to repress the activity of enzymes required for substrate metabolism. Accumulation of toxic metabolites may also repress further metabolic activity.

Ample evidence exists that some chemicals require the activities of several different groups of microorganisms for complete mineralization. Such consortia may not be found in the system containing the substrate. The interactions of these microorganisms pose additional constraints regarding production and utilization of potentially toxic metabolites as well as competition for nutrients and growth factors.

Finally, the dynamics of pollutant appearance in the system is of critical importance. Most microorganisms require a period of acclimation to the substrate before metabolism occurs. During this period, the substrate level must be high enough to promote acclimation without being toxic or inhibitory. Prior exposure to the compound helps to shorten the acclimation period. Such exposure to other pollutants may also predispose the microbial population to adaptation to the substrate of interest. Or such acclimation may result in destruction of microorganisms capable of substrate utilization in favor of a population adapted to a different substrate, or in virtually complete destruction of the microbial flora.

This review of microbiological decomposition of chlorinated aromatic compounds indicates that while certain metabolic pathway generalizations exist, as reflected in Figures 62, 63, and 64, biodegradation of the compounds shown is dependent on many other variables and cannot be assumed in any given biotic system. Data on many of these variables are needed to allow prediction of the metabolic fate of chlorinated aromatic compounds.

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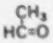
APPENDIX
ILLUSTRATED LIST OF COMPOUNDS

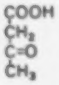
1.



$$\begin{array}{l}\mathrm{CH_3}\\ \mathrm{HC{=}O}\end{array}$$

ACETALDEHYDE

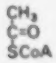
2.



$$\begin{array}{l}\mathrm{COOH}\\ \mathrm{CH_2}\\ \mathrm{C{=}O}\\ \mathrm{CH_3}\end{array}$$

ACETOACETIC ACID

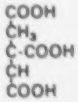
3.



$$\begin{array}{l}\mathrm{CH_3}\\ \mathrm{C{=}O}\\ \mathrm{SCoA}\end{array}$$

ACETYL-CoA

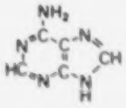
4.



$$\begin{array}{l}\mathrm{COOH}\\ \mathrm{CH_2}\\ \mathrm{C{-}COOH}\\ \mathrm{CH}\\ \mathrm{COOH}\end{array}$$

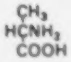
ACONITIC ACID

5.



ADENINE

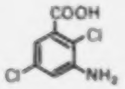
6.



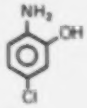
$$\begin{array}{l}\mathrm{CH_3}\\ \mathrm{HCNH_2}\\ \mathrm{COOH}\end{array}$$

ALANINE

7.

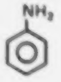


AMIBEN (chloramben, 3-amino-2,5-dichlorobenzoic acid)



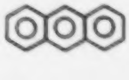
2-AMINO-5-CHLOROPHENOL

9.



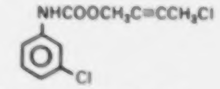
ANILINE

10.



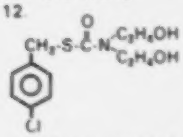
ANTHRACENE

11.



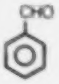
BARBAN [(3-chlorophenyl)-carbamic acid 4-chloro-2-butynyl ester]

12.



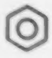
BENTHIOCARB (S-4-chlorobenzyl-N,N-diethylthiolcarbamate)

13.



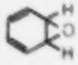
BENZALDEHYDE

14.



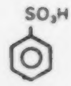
BENZENE

15.



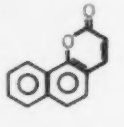
BENZENE 1,2-OXIDE

16



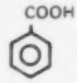
BENZENESULFONIC ACID

17



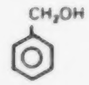
7,8-BENZOCOUMARIN

18



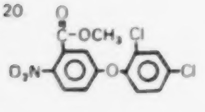
BENZOIC ACID

19



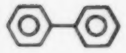
BENZYL ALCOHOL

20


O
C-OCH$_3$
Cl
O$_2$N
O
Cl

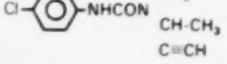
BIFENOX [methyl 5-(2,4-dichlorophenoxy)-2-nitrobenzoate]

21



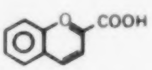
BIPHENYL

22


Cl
NHCON
CH$_3$
CH-CH$_3$
C≡CH

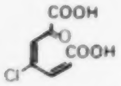
BUTURON[3-(4-chlorophenyl)-1-isobutynyl-1-methylurea]

23



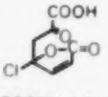
2-CARBOXYBENZOPYRILIUM

24


COOH
O
COOH
Cl

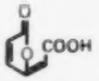
7-CARBOXY-4-CHLORO-2-KETO-HEPT-3,5-DIENOIC ACID

25


COOH
O
O
C=O
Cl

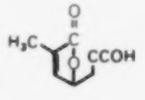
7-CARBOXY-4-CHLORO-2-KETO-HEPT-4,7-LACTONE

26



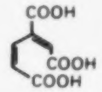
4-CARBOXYMETHYLENEBUT-2-EN-4-OLIDE

27


O
C
H$_3$C
O
CCOH

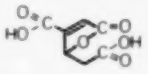
4-CARBOXYMETHYLENE-2-METHYL-Δ-BUTENOLIDE

28


COOH
COOH
COOH

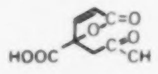
3-CARBOXY-cis-cis-MUCONIC ACID

29


O
C
HO
O
C=O
OH
C=O

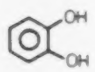
3-CARBOXYMUCONOLACTONE

30


O
C=O
O
C
CH
HOOC

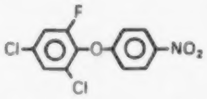
4-CARBOXYMUCONOLACTONE

31.



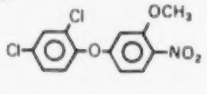
CATECHOL

32.



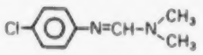
CFNP (2,4-dichloro-6-fluorophenyl-4'-nitrophenyl ether)

33.


Cl
OCH_3
Cl
O
NO_2

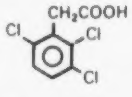
CHLOMETHOXYNIL (2,4-dichlorophenyl-3'-methoxy-4'-nitrophenyl ether)

34.


Cl
N=CH-N
CH_3
CH_3

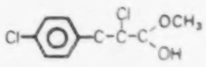
CHLORDIMEFORM[N-(4-chloro-o-tolyl)-N',N'-dimethylformamidine]

35.


CH_2COOH
Cl
Cl
Cl

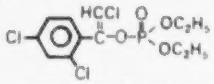
CHLORFENAC (fenac, 2,3,6-trichlorophenylacetic acid)

36.


Cl
C-C-C
Cl
OCH_3
OH

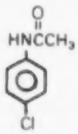
CHLORFENPROP METHYL
[methyl 2-chloro-3-(4-chlorophenyl) propionate]

37.


HCCl
O
Cl
C-O-P
OC_2H_5
OC_2H_5
Cl

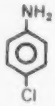
CHLORFENVINPHOS
2-chloro-1-(2,4-dichlorophenyl)-vinyl diethyl phosphate]

38.



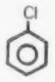
4-CHLOROACETANILIDE

39.



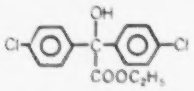
4-CHLOROANILINE

40.



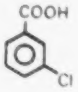
CHLOROBENZENE

41.


OH
Cl
C
Cl
$COOC_2H_5$

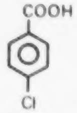
CHLOROBENZILATE
(ethyl 4,4'-dichlorobenzilate)

42.



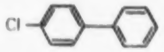
3-CHLORO-BENZOIC ACID

43.



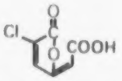
4-CHLOROBENZOIC ACID

44.



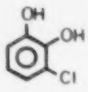
4-CHLOROBIPHENYL

45.



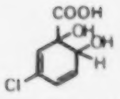
2-CHLORO-4-CARBOXY-METHYLENE-BUT-2-ENOLIDE

46.



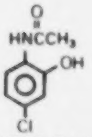
3-CHLOROCATECHOL

51.


COOH
OH
OH
H
Cl

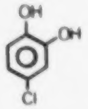
5-CHLORO-3,5-CYCLOHEXADIENE
1,2-DIOL-1-CARBOXYLIC ACID

56.


O
HNCCH₃
OH
Cl

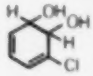
4-CHLORO-2-HYDROXY-
ACETANILIDE

47.



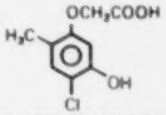
4-CHLOROCATECHOL

52.



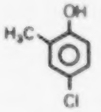
3-CHLORO-1,2-DIHYDROXY-
CYCLOHEXA-3,5-DIENE

57.


OCH₂COOH
H₃C
OH
Cl

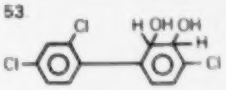
(4-CHLORO-5-HYDROXY-
2-METHYLPHENOXY)-
ACETIC ACID

48.



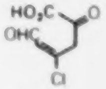
5-CHLORO-o-CRESOL

53.


Cl
H OH OH
H
Cl
Cl

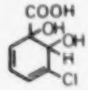
1-CHLORO-2,3-DIHYDROXY-
4-(2,4-DICHLOROPHENYL)-
HEXA-4,6-DIENE

58.


HO₂C
O
OHC
Cl

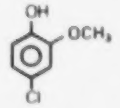
4-CHLORO-2-HYDROXY-
MUCONIC SEMIALDEHYDE

49.


COOH
OH
OH
H
Cl

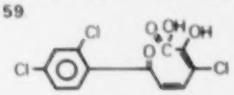
3-CHLORO-3,5-CYCLOHEXADIENE
1,2-DIOL-1-CARBOXYLIC ACID

54.



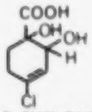
4-CHLOROGUAIACOL

59.


Cl
O OH
O C
OH
Cl
Cl

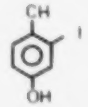
3-CHLORO-2-HYDROXY-6-(2,
4-DICHLOROPHENYL) HEXA-2,
4-DIENOIC ACID

50.


COOH
OH
OH
H
Cl

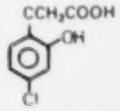
4-CHLORO-3,5-CYCLOHEXADIENE
1,2-DIOL-1-CARBOXYLIC ACID

55.



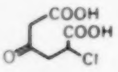
CHLOROHYDROQUINONE

60.


CCH₂COOH
OH
Cl

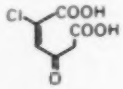
4-CHLORO-2-HYDROXY-
PHENOXYACETIC ACID

61.


COOH
COOH
O
Cl

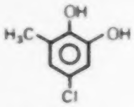
2-CHLORO-4-KETOADIPIC ACID

62.


Cl
COOH
COOH
O

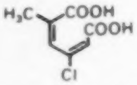
2-CHLOROMALEYLACETIC ACID

63.



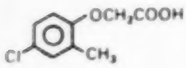
5-CHLORO-
3-METHYLCATECHOL

64.


H_3C
COOH
COOH
Cl

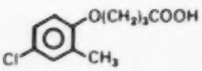
cis,cis-4-CHLORO-
2-METHYL-
MUCONIC ACID

65.


OCH_2COOH
Cl
CH_3

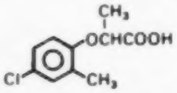
MCPA
(4-CHLORO-2-METHYL
PHENOXYACETIC ACID)

66.


$O(CH_2)_3COOH$
Cl
CH_3

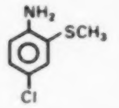
MCPB [4-(4-CHLORO-
2-METHYLPHENOXY)
BUTYRIC ACID]

67.


CH_3
OCHCOOH
Cl
CH_3

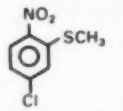
2-(4-CHLORO-2-METHYL
PHENOXY)PROPIONIC ACID

68.



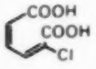
4-CHLORO-2-METHYL-
THIOBENZENEAMINE

69.



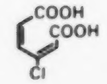
4-CHLORO-2-METHYLTHIO-
1-NITROBENZENE

70.


COOH
COOH
Cl

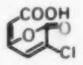
2-CHLOROMUCONIC ACID

71.


COOH
COOH
Cl

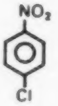
3-CHLOROMUCONIC ACID

72.



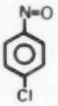
2-CHLOROMUCONOLACTONE

73.



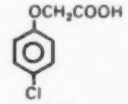
4-CHLORONITROBENZENE

74.



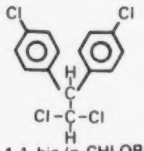
4-CHLORONITROSO-
BENZENE

75.


OCH_2COOH
Cl

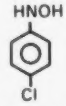
4-CHLOROPHENOXY-
ACETIC ACID

76.



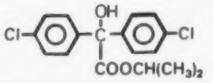
1,1-bis (p-CHLORO-
PHENYLETHANE)

77.



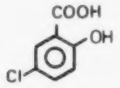
4-CHLOROPHENYL-
HYDROXYLAMINE

78.



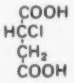
CHLOROPROPYLATE
(isopropyl 4,4′-dichlorobenzilate)

79.



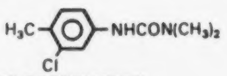
5-CHLOROSALICYLIC ACID

80.



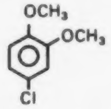
CHLOROSUCCINIC ACID

81.



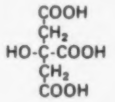
CHLOROTOLURON
(3-(3-chloro-4-methylphenyl)-
1,1-dimethylurea)

82.



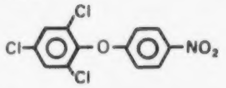
4-CHLOROVERATROLE

83.



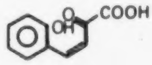
CITRIC ACID

84.



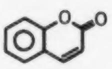
CNP
(2,4,6-TRICHLOROPHENYL-
4′-NITROPHENYL ETHER)

85.



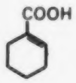
COUMARALDEHYDE

86.



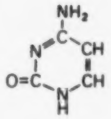
COUMARIN

87.



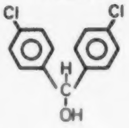
CYCLOHEX-1-ENE-
1-CARBOXYLIC ACID

88.



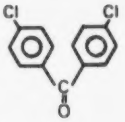
CYTOSINE

89.



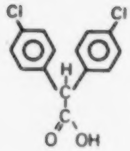
DBH
(DICHLOROBENZHYDROL)

90.



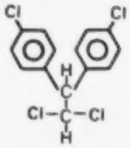
DBP
(DICHLOROBENZOPHENONE)

91.



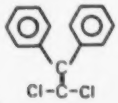
DDA
[2,2-bis(p-CHLOROPHENYL)
ACETIC ACID]

92.



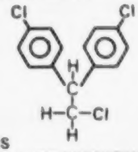
DDD
[1,1-DICHLORO-2,2-bis
(p-CHLOROPHENYL)ETHANE]

93.



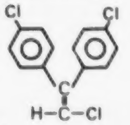
DDE
[2,2-bis(p-CHLOROPHENYL)-
1,1-DICHLOROETHYLENE]

94.



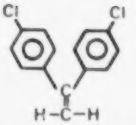
DDMS
[1,1-bis(p-CHLOROPHENYL-
2-CHLOROETHANE)]

95.



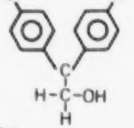
DDMU
[1-CHLORO-2,2-bis(p-CHLORO-
PHENYL)ETHYLENE]

96.



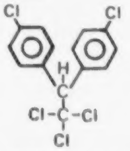
DDNU
[unsym-bis(p-CHLOROPHENYL)
ETHYLENE]

97.



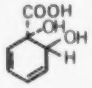
DDOH
[2,2-bis(p-CHLOROPHENYL)-
ETHANOL]

98.



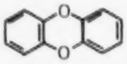
DDT
[1,1,1-TRICHLORO-2,2-bis-
(p-CHLOROPHENYL)ETHANE]

99.



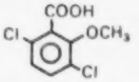
DHB
(3,5-CYCLOHEXADIENE-
1,2-DIOL-1-
CARBOXYLIC ACID)

100.



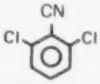
DIBENZO-p-DIOXIN

101.



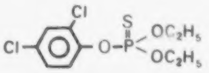
DICAMBA (3,6-DICHLORO-
o-ANISIC ACID)

102.



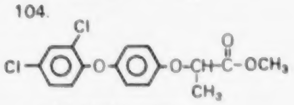
DICHLOBENIL
(2,6-DICHLOROBENZONITRILE)

103.



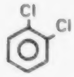
DICHLOFENTHION
[o,o-DIETHYL-o-
(2,4-DICHLOROPHENYL)-
PHOSPHOROTHIOATE]

104.



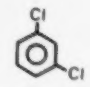
DICHLORFOP METHYL
[(±)-METHYL 2-[4-(2,4-DI-
CHLOROPHENOXY)PHENOXY]-
PROPIONATE]

105.



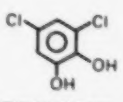
1,2-DICHLOROBENZENE

106.



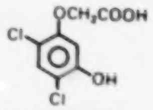
1,3-DICHLOROBENZENE

111.



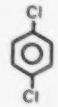
3,5-DICHLOROCATECHOL

116


OCH₂COOH
Cl
OH
Cl

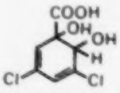
(2,4-DICHLORO-5-HYDROXYPHENOXY)-ACETIC ACID

107.



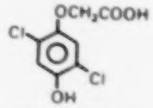
1,4-DICHLOROBENZENE

112.


COOH
OH
OH
H
Cl
Cl

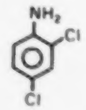
3,5-DICHLORO-3,5-CYCLOHEXADIENE-1,2-DIOL-1-CARBOXYLIC ACID

117


OCH₂COOH
Cl
Cl
OH

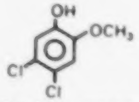
(2,5-DICHLORO-4-HYDROXYPHENOXY)-ACETIC ACID

108.



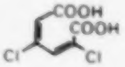
2,4-DICHLORO-BENZENEAMINE

113.


OH
OCH₃
Cl
Cl

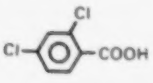
4,5-DICHLOROGUAIACOL

118.


COOH
COOH
Cl
Cl

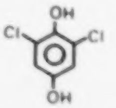
DICHLOROMUCONIC ACID

109.



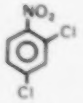
2,4-DICHLOROBENZOIC ACID

114.



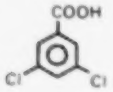
DICHLOROHYDROQUINONE

119.



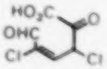
2,4-DICHLORO-1-NITROBENZENE

110.



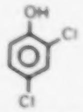
3,5-DICHLORO-BENZOIC ACID

115.


HO₂C
O
OHC
Cl
Cl

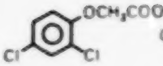
3,5-DICHLORO-2-HYDROXYMUCONIC SEMIALDEHYDE

120.



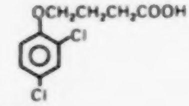
2,4-DICHLOROPHENOL (2,4-DCP)

121.


OCH₂COOH
Cl
Cl

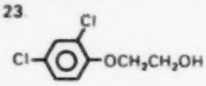
(2,4-DICHLORO-
PHENOXYACETIC ACID)

122.


OCH₂CH₂CH₂COOH
Cl
Cl

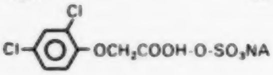
4-(2,4-D)B
4-(2,4-DICHLOROPHENOXY)
BUTYRIC ACID

123.


Cl
Cl
OCH₂CH₂OH

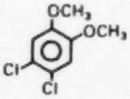
(2,4-DICHLOROPHENOXY)
ETHANOL

124.


Cl
Cl
OCH₂COOH-O-SO₃NA

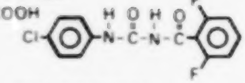
2-(2,4-DICHLOROPHENOXY)
ETHYL SULFATE,
SODIUM SALT

125.


OCH₃
OCH₃
Cl
Cl

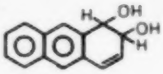
4,5-DICHLOROVERATROLE

126.


F
H O H O
Cl N C N C
F

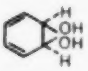
DIFLUBENZURON
[1-(4-CHLOROPHENYL)-
3-(2,6-DIFLUOROBENZOYL)UREA]

127.



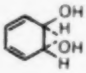
cis 1,2-DIHYDRO-1,2-
DIHYDROXYANTHRACENE

128.



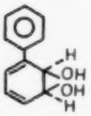
cis-1,2-DIHYDRO-
1,2-DIHYDROXYBENZENE

129.



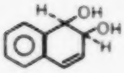
trans-1,2-DIHYDRO-1,2-
DIHYDROXYBENZENE

130.



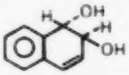
cis-2,3-DIHYDRO-
2,3-DIHYDROXYBIPHENYL

131.



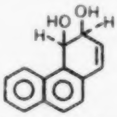
cis-1,2-DIHYDRO-1,2-
DIHYDROXYNAPHTHALENE

132.



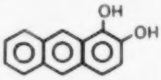
trans-1,2-DIHYDRO-
1,2-DIHYDROXYNAPHTHALENE

133.



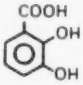
cis-3,4-DIHYDRO-3,4-
DIHYDROXYPHENANTHRENE

134.



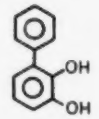
1,2-DIHYDROXY-
ANTHRACENE

135.



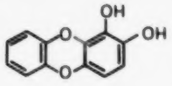
2,3-DIHYDROXYBENZOIC ACID

136.



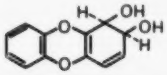
2,3-DIHYDROXYBIPHENYL

137.



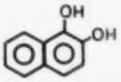
1,2-DIHYDROXY-
DIBENZO-p-DIOXIN

138.



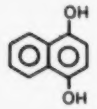
1,2-DIHYDROXY-
1,2-DIHYDRODIBENZO-p-DIOXIN

139.



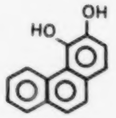
1,[illegible]-DIHYDROXYNAPHTHALENE

140.



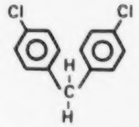
1,4-DIHYDROXYNAPHTHALENE

141.



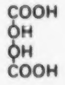
3,4-DIHYDROXYPHENANTHRENE

142.



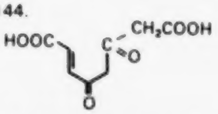
DPM
DICHLORODIPHENYLMETHANE

143.


COOH
OH
OH
COOH

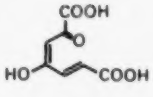
FUMARIC ACID

144.


CH₂COOH
HOOC
C
O
O

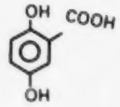
FUMARYLACETOACETIC
ACID

145.


COOH
O
HO
COOH

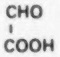
FUMARYLPYRUVIC ACID

146.



GENTISIC ACID

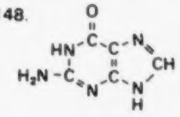
147.



$$\begin{array}{c}\mathrm{CHO}\\ |\\ \mathrm{COOH}\end{array}$$

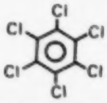
GLYOXYLIC ACID

148.


O
HN
C
C
N
CH
H₂N
C
N
C
N
H

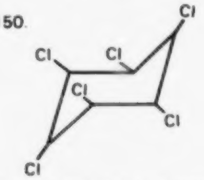
GUANINE

149.


Cl
Cl
Cl
Cl
Cl
Cl

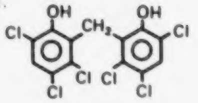
HEXACHLOROBENZENE

150.


Cl
Cl
Cl
Cl
Cl
Cl

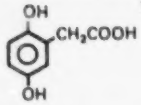
HEXACHLOROCYCLOHEXANE

151.



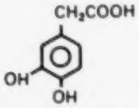
HEXACHLOROPHENE
[2,2'-METHYLENE bis-
(3,4,6-TRICHLOROPHENOL)]

152.



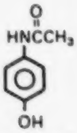
HOMOGENTISIC ACID

153.



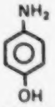
HOMOPROTOCATECHUIC ACID

154.



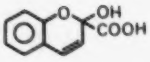
4-HYDROXYACETANILIDE

155.



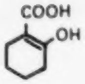
4-HYDROXYANILINE

156.



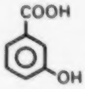
o-HYDROXYBENZALPYRUVIC ACID

157.



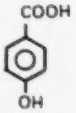
o-HYDROXYBENZOIC ACID

158.



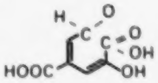
m-HYDROXYBENZOIC ACID

159.



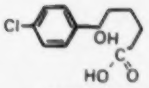
p-HYDROXYBENZOIC ACID

160.



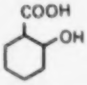
2-HYDROXY-4-CARBOXY-
MUCONIC SEMIALDEHYDE

161.



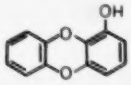
6-HYDROXY-6-
(4'CHLOROPHENYL)-
HEXANOIC ACID

162.



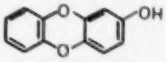
2-HYDROXY-
CYCLOHEXANE-
CARBOXYLIC ACID

163.



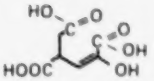
1-HYDROXY-
DIBENZO-p-
DIOXIN

164.



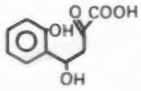
2-HYDROXY-
DIBENZO-p-
DIOXIN

165.



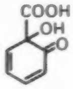
2-HYDROXY-4-CARBOXY-
MUCONIC ACID

166.



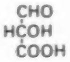
4-HYDROXY-1-o-HYDROXYPHENYL-2-OXOBUTYRIC ACID

167.



α-HYDROXY-β-KETOCARBOXYLIC ACID

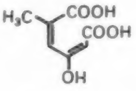
168.



$$\begin{matrix}\mathrm{CHO}\\ \mathrm{HCOH}\\ \mathrm{COOH}\end{matrix}$$

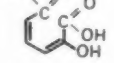
HYDROXYMALONIC SEMIALDEHYDE

169.



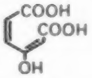
4-HYDROXY-2-METHYL-MUCONIC ACID

170.



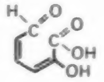
2-HYDROXYMUCONIC ACID

171.



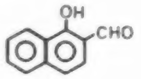
3-HYDROXYMUCONIC ACID

172.



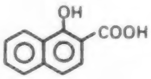
2-HYDROXYMUCONIC SEMIALDEHYDE

173.



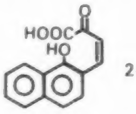
1-HYDROXY-2-NAPHTHALDEHYDE

174.



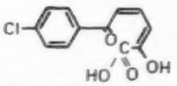
1-HYDROXY-2-NAPHTHOIC ACID

175.



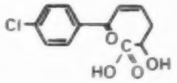
cis-4- -HYDROXY-NAPHTH-2-YL -2-OXOBUT-3-ENOIC ACID

176.



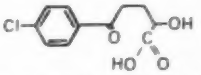
2-HYDROXY-6-OXO-6-(4'-CHLOROPHENYL) HEXA-2, 4-DIENOIC ACID

177.



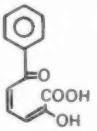
2-HYDROXY-6-OXO-6-(4'-CHLOROPHENYL)-4-HEXENOIC ACID

178.



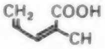
2-HYDROXY-5-OXO-5-(4'-CHLOROPHENYL-PENTANOIC ACID

179



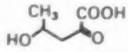
2-HYDROXY-6-OXO-6-PHENYLHEXA-2,4-DIENOIC ACID

180.



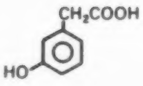
2-HYDROXYPENTA-2,4-DIENOIC ACID

181.



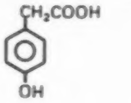
4-HYDROXY-2-OXOVALERIC ACID

182.



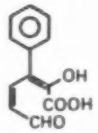
3-HYDROXYPHENYLACETIC ACID

183.



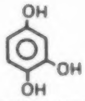
4-HYDROXYPHENYLACETIC ACID

184.



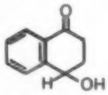
2-HYDROXY-3-PHENYLMUCONIC SEMIALDEHYDE

185.



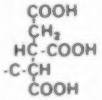
HYDROXYQUINOL

186.



4-HYDROXY-1-TETRALONE

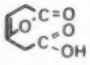
187.



$$\mathrm{COOH{-}CH_2{-}HC(COOH){-}CH({-}C{-})COOH}$$

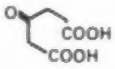
ISOCITRIC ACID

188.



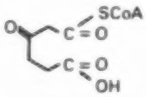
3-KETOADIPATE ENOL-LACTONE

189.



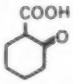
β-KETOADIPIC ACID

190.



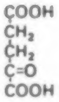
3-KETOADIPYL CoA

191.



2-KETO-CYCLOHEXANE-CARBOXYLIC ACID

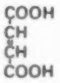
192.



$$\mathrm{COOH{-}CH_2{-}CH_2{-}C{=}O{-}COOH}$$

2-KETOGLUTARIC ACID

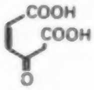
193.



$$\mathrm{COOH{-}CH{=}CH{-}COOH}$$

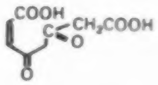
MALEIC ACID

194.



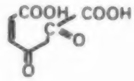
MALEYL-ACETIC ACID

195.



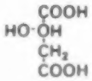
MALEYLACETOACETIC ACID

196.



MALEYLPYRUVIC ACID

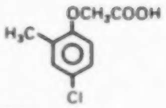
197.



$$\mathrm{COOH{-}OH(HO){-}CH_2{-}COOH}$$

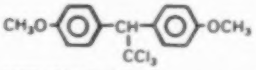
MALIC ACID

198



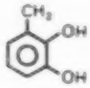
MCPA
(4-CHLORO-2-METHYL-
PHENOXYACETIC ACID)

199



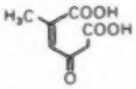
METHOXYCHLOR
[2,2-*bis* (*p*-methoxyphenyl)-1,1,1-
trichloroethane]

200



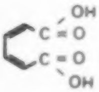
3-METHYLCATECHOL

201



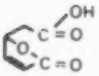
3-METHYLMALEYL ACETATE

202



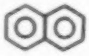
MUCONIC ACID

203



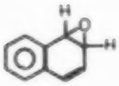
MUCONOLACTONE

204



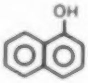
NAPHTHALENE

205



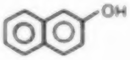
NAPHTHALENE 1,2-OXIDE

206



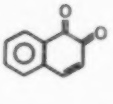
1-NAPHTHOL

207



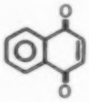
2-NAPHTHOL

208



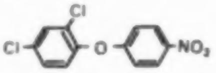
1,2-NAPHTHOQUINONE

209



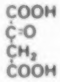
1,4-NAPHTHOQUINONE

210



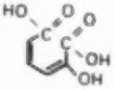
NITROFEN
(2,4-DICHLOROPHENYL-
4'-NITROPHENYL ETHER)

211



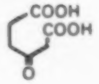
OXALOACETIC ACID

212



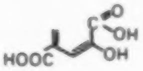
4-OXALOCROTONIC ACID

213.



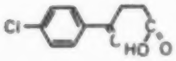
3-OXOADIPIC ACID

214.


C=O
OH
HOOC
OH

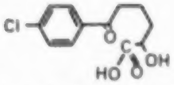
2-OXO-4-CARBOXYPENT-4-ENOATE

215.



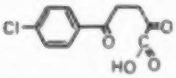
4-OXO-4-(4'-CHLOROPHENYL)-BUTANOIC ACID

216.



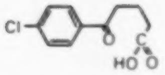
6-OXO-6-(4'-CHLOROPHENYL)-2-HYDROXYHEXANOIC ACID

217.



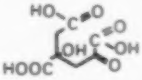
2,5-OXO-5-(4'-CHLOROPHENYL)-PENTANOIC ACID

218.



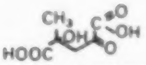
5-OXO-5-(4'-CHLOROPHENYL)-PENTANOIC ACID

219.


HO
C=O
C=O
OH
OH
HOOC
O

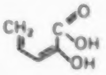
2-OXO-4-HYDROXY-4-CARBOXYMUCONIC ACID

220.


CH₃
C=O
OH
OH
HOOC
O

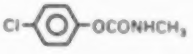
2-OXO-4-HYDROXY-CARBOXYPENTANOIC ACID

221.



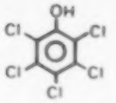
2-OXOPENT-4-ENOIC ACID

222.


Cl
OCONHCH₃

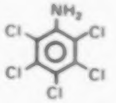
PCMC
(4-CHLOROPHENYL
N-METHYLCARBAMATE)

223.


OH
Cl
Cl
Cl
Cl
Cl

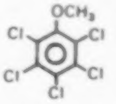
PCP
(PENTACHLOROPHENOL)

224.


NH₂
Cl
Cl
Cl
Cl
Cl

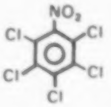
PENTACHLOROANILINE

225.


OCH₃
Cl
Cl
Cl
Cl
Cl

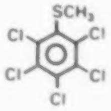
PENTACHLOROANISOLE

226.


NO₂
Cl
Cl
Cl
Cl
Cl

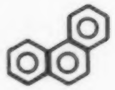
PENTACHLORONITROBENZENE

227.


SCH₃
Cl
Cl
Cl
Cl
Cl

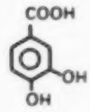
PENTACHLOROTHIOANISOLE

228.



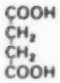
PHENANTHRENE

233.



PROTOCATECHUIC ACID

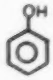
238.



$\mathrm{COOH{-}CH_2{-}CH_2{-}COOH}$

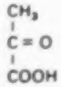
SUCCINIC ACID

229.



PHENOL

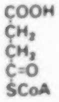
234.



$\mathrm{CH_3{-}C{=}O{-}COOH}$

PYRUVIC ACID

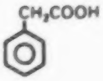
239.



$\mathrm{COOH{-}CH_2{-}CH_2{-}C{=}O{-}SCoA}$

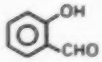
SUCCINYL-CoA

230.



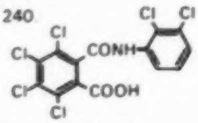
PHENYLACETIC ACID

235.



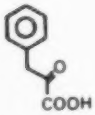
SALICYLALDEHYDE

240.



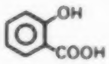
TECHLOFTHALAM
[N-(2,3-dichlorophenyl)-3,4,
5,6-tetrachlorophthalamic acid]

231.



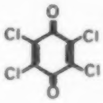
PHENYLPYRUVIC ACID

236.



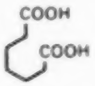
SALICYLIC ACID

241.



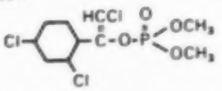
TETRACHLOROBENZOQUINONE

232.



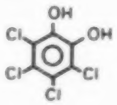
PIMELIC ACID

237.



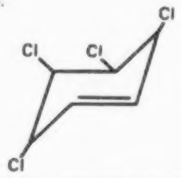
SD 8280
[2-chloro-1-(2,4-dichlorophenyl)-
vinyl dimethyl phosphate]

242.



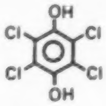
TETRACHLOROCATECHOL

243.



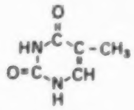
TETRACHLOROCYCLOHEXENE

244.



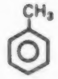
TETRACHLOROHYDROQUINONE

245.



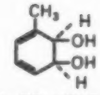
THYMINE

246.



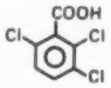
TOLUENE

247.



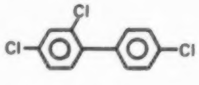
cis-TOLUENE
DIHYDRODIOL

248.



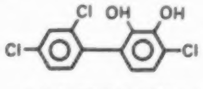
2,3,6-TRICHLORO-
BENZOIC ACID

249.



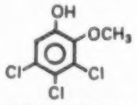
2,4,4′-TRICHLOROBIPHENYL

250.



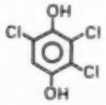
2,4,4′-TRICHLORO-2′,
3′-DIHYDROXYBIPHENYL

251.



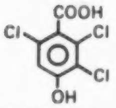
3,4,5-TRICHLOROGUAIACOL

252.



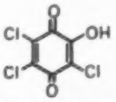
TRICHLOROHYDROQUINONE

253.



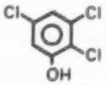
2,3,6-TRICHLORO-
4-HYDROXYBENZOIC ACID

254.



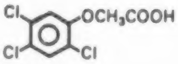
TRICHLOROHYDROXY-
BENZOQUINONE

255.



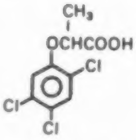
2,3,5-TRICHLORO-
PHENOL

256.



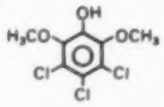
2,4,5-T
(2,4,5-TRICHLORO-
PHENOXYACETIC ACID)

257.



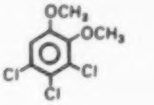
2-(2,4,5-TRICHLOROPHENOXYPRO-
PIONIC ACID
(SILVEX)

258.


OH
H₃CO
OCH₃
Cl
Cl
Cl

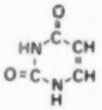
3,4,5-TRICHLOROSYRINGOL

259.


OCH₃
OCH₃
Cl
Cl
Cl

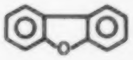
3,4,5-TRICHLOROVERATROLE

260.


O
HN
C
CH
O=C
N
CH
H

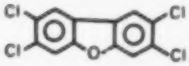
URACIL

261.



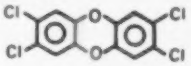
DIBENZOFURAN

262.



2,3,7,8,-TETRACHLORODIBENZOFURAN

263.


Cl
O
Cl
Cl
O
Cl

2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN

GLOSSARY

Acidophile: Organism that grows very well at an acidic pH.

Active transport: Process in which substrate entry into a cell is coupled to an energy-yielding process.

Adenine: Purine base unit of a nucleoside.

Allosteric enzyme: Enzyme that contains a regulatory site.

Anaerobic respiration: Oxidative process similar to aerobic respiration but which utilizes nitrate or another inorganic compound as the terminal electron acceptor.

ATP (adenosine triphosphate): Ribonucleoside 5′-triphosphate that serves as a phosphate-group donor in the cell's energy cycle.

Autoradiograph: Picture formed on film resulting from exposure to radioactive particles.

Autotroph: Organism that obtains its energy from the oxidation of organic or inorganic compounds.

Bacteriophage: Virus that attacks bacteria.

Barophilic: Requiring high barometric pressures for survival.

Barotolerant: Ability to survive at a wide range of barometric pressures.

Basophile: Organism that grows at alkaline pH.

Binary fission: Process of cell replication whereby a single cell divides into two.

Budding: Process of cell replication whereby a protruberance from a cell grows into another cell.

Capsule: Extracellular polysaccharide which accumulates around the cell and functions in cell attachment, defense, and protection.

Carboxylase: Enzyme that catalyzes the ATP-dependent addition of carbon dioxide to the acceptor substrate.

Catabolite repression: Inhibition of enzyme activity by binding of a control protein to the operator site.

Cell wall: Outer portion of bacterial cell that confers upon the cell its shape.

Chemostat: An apparatus used for the continuous culture of microbial populations in a steady state in which the growth rate is maintained by the substrate dilution rate.

Chemotaxis: Movement in response to a specific chemical.

Chemotroph: See autotroph.

Chimera: Molecule consisting of a replicon and another fragment of DNA.

Chromosome: A single large molecule of DNA that contains many genes.

Codon: A group of three adjacent nucleotides that codes for an amino acid.

Cometabolism: Process by which a substrate is metabolized by a cell while the cell utilizes another substrate as its energy source.

Competency: State in which a cell is able to undergo transformation.

Competitive inhibitor: Chemical which has a similar structure to an enzyme substrate and therefore binds to the enzyme, but which does not activate the enzyme.

Complete-mix reactor: A reactor in which, at any instant in time, the concentration of constituents is the same at any point in the reactor. The effluent concentration of a constituent is therefore also the same as the concentration in the reactor.

Conjugation: Process of DNA transfer from one bacterial cell to another by direct cell to cell contact.

Conjugative plasmid: Plasmid that contains genes for bacterial conjugation.

Consortia: Mixtures of different populations.

Continuous stirred tank reactor (CSTR): A reactor in which mechanical agitation is used to generate a complete-mix condition where, at any instant in time, the concentration of constituents is the same at any point in the reactor. The effluent concentration of a constituent is therefore also the same as the concentration in the reactor.

Copy number: The number of copies of a single plasmid present within a single cell.

Cosmid: Constructed vector used for cloning large fragments of DNA.

Cytochrome P-450: Protein that serves as an electron carrier in enzymatic hydroxylation reactions and can also transfer electrons to oxygen.

Cytoplasmic membrane: The limiting boundary of the cell protoplasm, composed of protein and phospholipid, that functions in substrate transport, osmotic regulation, cell wall synthesis, oxidative metabolism and energy production.

Cytosine: Pyrimidine base unit of a nucleoside.

Death phase: Period in which reduction in population occurs due to cell death.

Decarboxylase: Enzyme that catalyzes decarboxylation of the substrate.

Dehydrogenase: Enzyme that mediates the loss of a hydrogen ion from a substrate with the acceptor being other than molecular oxygen.

Denaturation: Process of separating double-stranded DNA into single strands.

Deoxyribonuclease: Enzyme that catalyzes random cleavage of double-stranded or single-stranded DNA.

Deoxyribonucleic acid (DNA): A polynucleotide consisting of deoxyribonucleotide units that serves as the carrier of genetic information.

Deterministic: Use of knowledge of the causes of a process to arrive at a prediction of its performance.

Dioxygenase: Enzyme that catalyzes the addition of two atoms of molecular oxygen to a molecule.

Enzyme: Protein which both lowers the energy of activation of and directs the metabolic pathway taken by chemical reactions in an organism.

Eukaryote: Organism characterized by having a nucleus surrounded by a membrane.

Exogenous DNA: DNA molecule which is not an integral part of the cell genome.

Exonuclease: Enzyme that removes single nucleotides from the end of a DNA molecule.

Exponential phase (Log phase): Growth phase during which cells divide by binary fission.

Extracellular: Outside of the outermost layer of a cell (cell membrane or cell wall).

Extracellular water: The water in biomass "solids" residing between cells. Compounds residing in extracellular water are not included in measures of passive or active cellular uptake.

Facultative anaerobe: Organism that grows in the presence or absence of air.

Feedback inhibition: Inhibition of an allosteric enzyme early in a metabolic pathway by a later product of the pathway.

Fermentation pathway: Metabolic pathway in which organic compounds serve as both the electron donor and the electron acceptor.

Fiber wall reactor: A reactor in which the biomass is contained within a fibrous inner cavity. The aqueous solution and its dissolved constituent are thus permitted to pass through the fiber well but the biomass (fixed film and suspended) is contained within the inner cavity. This type of reactor obviates the need for biomass separation by settling and recycle to the system.

Fill-and-draw reactor: A mode of operating a reactor in which reactants and products are added or removed over discrete time intervals but the reactions are allowed to proceed continuously. Since concentrations of constituents change cyclically during the test, results of this type of test may only approximate true continuous and steady-state tests.

Flagellum: A hairlike organelle attached to a cell that functions in motility.

Freundlich isotherm: An isotherm equation relating the equilibrium partitioning of a compound between liquid and solid compartments (or phases). The equation is of the form

$$C_s = K_f C_l^{1/n}$$

where C_s and C_l are concentrations of the compound in solid and liquid compartments, respectively, and K_f and n are empirical constants.

Gene: A DNA segment that codes for a single polypeptide chain or RNA molecule.

Genetic recombination: Process of combining DNA from different sources into a molecule.

Genome: The entire group of genes of a cell.

Gram negative: Term given to bacteria that lose the primary stain (crystal violet) of the Gram staining procedure upon exposure to alcohol or other decolorizing agent.

Gram positive: Term given to bacteria that retain the primary stain (crystal violet) of the Gram staining procedure upon exposure to alcohol or other decolorizing agent.

Gram stain: Differential staining procedure in which crystal violet, Gram's iodine, decolorizing agent such as alcohol, and safranin are sequentially applied to bacterial cells. Most bacteria can be divided into two groups based on whether they retain or lose the primary stain (crystal violet) during the procedure. The response of bacterial cells to this procedure has been linked to differences in the cell wall composition.

Guanine: Purine base unit of a nucleoside.

Halophile: Organism that requires high salt concentrations for growth.

Henry's law constant: A constant that describes the equilibrium partitioning of a compound between liquid and gas compartments (or phases) at a given condition, where the concentration of the compound in the liquid compartment is sufficiently low. Many units are possible with this constant and caution should be taken in its use.

Heterotroph: Organism that requires an organic form of carbon for energy.

Holdfast: Appendage of some bacteria consisting of a fine stalk which may possess adhesive material; functions in attachment.

Hybridization: Process of joining two nucleotides.

Hydraulic residence time (HRT): The time that the bulk aqueous phase resides in a continuous reactor volume. This may be calculated as the ratio of the system's hydraulic volume and the flow rate of the aqueous feed stream, assuming no change in fluid density during the reaction.

Hydrolase: Enzyme that mediates the transfer of a chemical group to water.

Hydrolysis: Cleavage of a molecule by reaction with water.

Hydrophilic: Water-loving; refers to polar molecules that associate with water.

Hydrophobic: Water-hating; refers to nonpolar molecules that are insoluble in water.

Hypha: A fungus thread.

Inducer: A molecule that induces the activity of an enzyme.

Insertion sequence: Segment of DNA occurring on either end of a transposon.

In situ: In its original position.

Intron: An intervening sequence in a gene that is transcribed but excised before translation of the gene.

In vitro: In a test tube or beaker ("in glass").

In vivo: In a living organism.

Irreversible inhibitor: Chemical that destroys or binds to a functional group on an enzyme, thereby preventing its catalytic activity.

Isomerase: Enzyme that catalyzes a change in the atomic configuration of a molecule without a change in the number or kind of atoms.

Jet drop entrainment: A mechanism in which small particles may be launched into a gaseous phase based on the collapsing of bubbles at the gas-liquid surface. Small droplets of fluid originating at the base of the bubble are formed and accelerated as the top of the bubble breaks and the bottom of the bubble merges with the gas-liquid surface. These drops may be aerosolized and thus carried with the gas phase.

3-Ketoadipate pathway: Aerobic pathway of aromatic compound dissimilation in which the end products are the tricarboxylic acid cycle intermediates succinate and acetyl-CoA.

Kinase: Enzyme that catalyzes transfer of a phosphate group from ATP or other nucleoside triphosphate to the substrate.

Lag phase: Growth phase during which adaptation to the environment occurs and no increase in cell number is seen.

Langmuir isotherm: An isotherm equation relating the equilibrium partitioning of a component between liquid and solid compartments (or phases). The equation is of the form:

$$C_s = \frac{ab\,C_L}{1 + b\,C_L}$$

where C_s and C_L are concentrations of the compound in solid and liquid compartments, respectively, and a and b are empirical constants. This isotherm may be derived from considerations of mass transport onto surfaces with limited capacity to sorb the compound.

Ligase: Enzyme that catalyzes the formation of a product resulting from the condensation of two different molecules, coupled with the cleavage of a pyrophosphate linkage in ATP.

Lipophilic: Refers to molecules that associate with lipids.

Lithotrophs: Bacteria that use inorganic compounds as substrates for respiratory metabolism.

Log phase: See exponential phase.

Lyase: Enzyme that catalyzes the addition of a chemical group to the double bond of a substrate or the removal of a chemical group to form a double bond.

Lyse: Breaking apart of the cell wall.

Lysogeny: Infection of a bacterial cell by a virus during which the viral genome becomes integrated into the cell genome, is repressed, and is replicated with the cell genome.

Lytic cycle: Process by which a bacteriophage infects a cell, replicates, and is released into the environment.

Macroinvertebrates: Group of organisms that lack a backbone; in this context refers to species that live in the water.

Maximum specific growth rate (μ_m): The specific growth rate of biomass on a substrate that is limiting growth as defined by the Monod equation:

$$\mu = \mu_m \frac{C_s}{K_s + C_s}$$

where μ is the specific growth rate, μ_m is the maximum growth rate measured, K_s is the Monod half-saturation constant ($K_s = C_s$ at a growth rate of $\mu_m/2$), and C_s is the concentration of the growth-limiting substrate.

Mean cell residence time (MCRT): The mean of the cellular time distribution descriptive for the reactor configuration under consideration. This equals the hydraulic residence time in complete-mix, suspended growth reactors that have no biomass separation and recycle. This is a design variable that may be determined in suspended growth reactor systems that have provision for biomass separation and recycle and is determined by biokinetic rate constants and physical design in fixed film systems.

Mesocosm: A constructed laboratory representation of an environment including atmospheric, hydrospheric, and geospheric parts with associated flora and fauna.

Mesophile: Organism that grows best at temperatures from 15°C to about 45°C.

Messenger RNA (mRNA): RNA molecule that serves to carry the genetic message from the DNA to the ribosome.

***Meta* position:** Position on an aromatic molecule separated from the point of reference by one carbon position.

Methylase: Enzyme that adds a methyl group to particular nucleotides.

Microaerophile: Organism that has a narrow range of tolerance for its gaseous environment and requires either a reduced air environment or, in some cases, an increased proportion of carbon dioxide.

Microcosm: A small-scale version of a mesocosm. See mesocosm.

Mineralize: Convert a molecule to inorganic ions and molecules.

Mitochondria: Organelles in a eukaryotic cell which are the sites of oxidative metabolism.

Mixed liquor: The mixture of the aqueous phase and suspended biomass in the aeration reactor of a biological treatment process.

Mixed liquor suspended solids (MLSS): A measure of biomass in suspended biological processes where solids are separated from the mixed liquor, are dried, and weights are determined gravimetrically. Standard methods exist for this test.

Mixed liquor volatile suspended solids (MLVSS): A measure of biomass in suspended biological processes where solids are separated from the mixed liquor, dried, heated to remove volatile organics, and weights are determined gravimetrically. Standard methods exist for the test.

Monod half-saturation constant (K_s): A constant defined in the Monod equation. See maximum specific growth rate.

Monooxygenase: Enzyme that catalyzes the addition of one atom of molecular oxygen to a molecule.

Mutant: Cell in which the genome has undergone mutation.

Mutase: Enzyme that catalyzes transfer of a functional group between two positions on the same molecule.

Mutation: Alteration of the genetic message.

NAD^+, NADH: Nicotinamide adenine dinucleotide, a coenzyme which functions in oxidation-reduction reactions as hydrogen and electron carriers.

$NADP^+$, NADPH: Nicotinamide adenine dinucleotide phosphate. Same function as NAD; see NAD.

Neutrophile: Organism that grows best at a neutral pH.

NIH shift: Migration of a hydrogen atom from one carbon to the adjacent carbon on an aromatic molecule.

Noncompetitive inhibitor: Chemical that binds to an enzyme in an area other than the binding site, thereby altering and inactivating the catalytic site.

Nucleoid: The area in a prokaryotic cell that contains the chromosome and is not bounded by a membrane.

Nucleoside: A compound composed of a purine or pyrimidine base covalently linked to a pentose sugar.

Nucleotide: A nucleoside with a phosphate group attached to one of the pentose hydroxyl groups.

Nucleus: The membrane-bound organelle in a eukaryotic cell that contains the chromosome.

Nick: A breakage in one strand of a double-stranded DNA molecule.

Obligate aerobe: Organism that grows in the presence of air and uses aerobic respiration to obtain energy.

Obligate anaerobe: Organism that grows only in the absence of air.

Octanol-water partition coefficient (K_{ow}): A constant that describes the equilibrium partitioning of a compound between equal volumes of n-octanol and water at a given temperature. Partitioning between other immiscible fluids and between other compartments can be mathematically related to this constant.

Operator region: Regulatory site of a gene.

Organelle: A discrete portion of a cell, with a specific function.

Organotrophs: Bacteria that use organic compounds as substrates for respiratory metabolism.

Origin of replication: Sequence of DNA required for replication of the molecule.

***Ortho* position:** Position on an aromatic molecule adjacent to the point of reference.

Osmotic shock: Sudden change in the solute concentration of the environment surrounding a cell.

Oxidase: Enzyme that catalyzes loss of a hydrogen ion with molecular oxygen as the acceptor.

Oxidoreductase: Enzyme that mediates alterations of the CH-OH group of a substrate and requires NAD^+ or $NADP^+$ as the hydrogen acceptor.

***Para* position:** Position on an aromatic molecule separated from the point of reference by two carbon positions, effectively opposite to the point of reference.

Passive transport: Process by which substrates enter a cell by free diffusion dependent on the difference in substrate concentration inside and outside of the cell.

Pathogenic: Capable of causing disease.

Periplasmic space: Area between the cytoplasmic membrane and the cell wall.

Phosphatase: Enzyme that mediates the hydrolytic cleavage of phosphate esters.

Phototroph: Organism that obtains its energy from light.

Phytotoxic: Capable of inhibiting the growth of plants or algae.

Plasmid: Small circular DNA molecule that is extrachromosomal and replicates autonomously.

Pleomorphic: Capable of changing shape.

Polylinker: Segment of DNA that contains closely spaced recognition sites for several restriction endonucleases.

Polymerase: Enzyme that adds nucleotides to the 3′-hydroxyl terminus or removes nucleotides from the 5′-phosphate terminus of nicked DNA.

Primary degradation: The initial alteration of a compound.

Primer: Short section of DNA required to be attached to mRNA to initiate the activity of reverse transcriptase.

Prokaryote: Organism characterized by lacking a nuclear membrane.

Protoplasm: The cell within the cell wall.

Protoplast: A viable cell that lacks a cell wall.

Psychrophile: Organism that grows best at temperatures below 20°C.

Recycling fermentor: An apparatus for the continuous culture of microorganisms in a steady state whereby the cells are returned to the culture vessel while medium and waste materials are removed.

Regulatory sequence: A DNA segment involved in regulating a gene.

Regulatory site: Area on an enzyme reversibly occupied by a noncompetitive inhibitor.

Relaxed plasmid: Plasmid which is present in a cell as multiple copies.

Replicon: DNA molecule that contains an origin of replication.

Repressor protein: Protein that binds to the operator region of a gene and blocks its transcription.

Respiration pathway: Metabolic pathway in which oxygen or other inorganic compound or ion serves as the terminal electron acceptor.

Resting cell: A viable cell which is not actively growing or dividing.

Restriction endonuclease: Enzyme that recognizes and cleaves specific sequences of nucleotides within double-stranded DNA.

Reverse transcriptase (RNA-dependent DNA polymerase): Enzyme that catalyzes the formation of double-stranded DNA from the information on mRNA.

Reversible inhibitor: Chemical that binds to an enzyme but which may be removed with resulting activation of the enzyme; see competitive inhibitor, noncompetitive inhibitor.

Ribonucleic acid (RNA): A polynucleotide composed of ribonucleotide units.

Ribosomal RNA (rRNA): RNA molecule attached to the ribosome that serves as a framework for the binding of the polypeptide subunits of a protein.

Ribosome: Site of protein biosynthesis.

RNA-dependent DNA polymerase: See reverse transcriptase.

RNA polymerase: Enzyme that catalyzes the formation of RNA from the information on DNA or RNA.

Semi-continuous reactors: Reactors that are operated by adding or removing reactant or products over discrete time intervals, but where the reactions are allowed to proceed continuously. See fill and draw reactor.

Sequential induction: Control of a long metabolic pathway such that sections of the pathway are under separate regulatory control and each section is induced by the product of a prior section.

Sludge volume index (SVI): A measure of settleability of suspended biomass that is based on the volume of solids settled from a mixed liquor over a given time interval. Standard methods exist for this test.

Stationary phase: Growth phase during which no net change in cell numbers is seen; number of cells generated and dying is equivalent.

Steady state: The condition where properties of a system of any given point in the system are the same over time.

Steric hindrance: The inability of atoms or groups on a molecule to rotate freely because of mutual repelling due to van der Waals forces.

Sticky end: Linear double-stranded DNA in which one strand extends beyond the other.

Stringent plasmid: Plasmid which is present in a cell in one or, at the most, three copies.

Structural gene: A gene that codes for a protein.

Suspended biomass: The state of biomass growth where sufficient mechanical energy is introduced by the cells or from external sources to favor free suspension of cells of flocs of biomass in the mixed liquor or broth and to avoid the formation of a fixed biofilm on reactor surfaces.

Synthetase: Enzyme that mediates condensation of two separate molecules coupled with cleavage of ATP.

T_4 DNA ligase: Enzyme that links together complementary fragments of double-stranded DNA.

T_4 RNA ligase: Enzyme that links together complementary fragments of single-stranded DNA or RNA.

Taxonomy: The science of arranging organisms into logical groups describing in detail the basic taxonomic unit, the species.

Terminal deoxynucleotidyl transferase: Enzyme that adds deoxynucleotides to the 3′-hydroxyl end of DNA.

Thermophile: Organism that grows at temperatures above 50°C.

Thiokinase: Enzyme that catalyzes the ATP-dependent formation of thiol esters.

Thylakoid: Internal membrane structure in cyanobacteria that contains the photosynthetic apparatus.

Thymine: Pyrimidine base unit of a deoxyribonucleoside.

Transcription: Process of converting information coded by DNA into RNA.

Transduction: Bacteriophage-mediated transfer of genetic material into a cell.

Transferase: Enzyme that catalyzes the transfer of an intact group of atoms from a donor to an acceptor molecule.

Transfer RNA (tRNA): RNA molecule that serves to bring a specific amino acid into proximity with the developing polypeptide.

Transformation: Process of transfer of exogenous DNA into a cell.

Translation: Process of protein biosynthesis according to the code carried by the mRNA.

Transposon: A segment of DNA that can be moved from one area on a chromosome to another.

Tricarboxylic acid cycle: Respiration pathway utilized by aerobic organisms.

Unsteady state: The condition where properties of a system at any given point in the system are changing over time.

Uracil: Pyrimidine base unit of a ribonucleoside.

Vector: A replicon to which a fragment of DNA may be attached so that the fragment may be replicated.

Vesicle: Cavity filled with liquid or gas.

Viable: Capable of growing.

Yield coefficient (Y): The ratio of the change in biomass concentration, X, and the change in substrate concentration, C_s, over an interval of time. This is a measure of biomass production for unit substrate removal.

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